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ORIGINAL ARTICLE

Common α2A and α2C adrenergic receptor polymorphisms do not affect plasma membrane trafficking

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Abstract Various naturally occurring polymorphic forms of human G protein-coupled receptors (GPCRs) have been identified and linked to diverse pathological diseases, including receptors for vasopressin type 2 (nephrogenic diabetes insipidus) and gonadotropin releasing hormone (hypogonadotropic hypogonadism). In most cases, polymorphic amino acid mutations disrupt protein folding, altering receptor function as well as plasma membrane expression. Other pathological GPCR variants have been found that do not alter receptor function, but instead affect only plasma membrane trafficking (e.g., delta opiate and histamine type 1 receptors). Thus, altered membrane trafficking with retained receptor function may be another mechanism causing polymorphic GPCR dysfunction. Two common human α2A and α2C adrenergic receptor (AR) variants have been identified (α2A N251K and α2C Δ322-325 ARs), but pharmacological analysis of ligand binding and second messenger signaling has not consistently demonstrated altered receptor function. However, possible alterations in plasma membrane trafficking have not been investigated. We utilized a systematic approach previously developed for the study of GPCR trafficking motifs and accessory proteins to assess whether these α2 AR variants affected intracellular trafficking or plasma membrane expression. By combining immunofluorescent microscopy, glycosidic processing analysis, and quantitative fluorescent-activated cell sorting (FACS), we demonstrate that neither variant receptor had altered intracellular localization, glycosylation, nor plasma membrane expression compared to wild-type α2 ARs. Therefore, pathopharmacological properties of α2A N251K and α2C Δ322-325 ARs do not appear to be due to altered receptor pharmacology or plasma membrane trafficking, but may involve interactions with other intracellular signaling cascades or proteins.

Keywords α2 adrenergic receptor · G protein-coupled receptor · Polymorphism · Quantitative FACS · Trafficking

Introduction

The large gene family of G protein-coupled receptors (GPCRs) has major roles in health and disease. GPCRs are the pharmacological site of action for many drugs, and naturally occurring human GPCR polymorphisms have been linked to various loss-of-function disorders in humans. Some examples of human diseases linked to variant forms of GPCRs include retinitis pigmentosa (rhodopsin (Dryja et al. 1990)), nephrogenic diabetes insipidus (vasopressin type 2 receptor (V2R) (Rosenthal et al. 1992)), and hypogonadotropic hypogonadism (gonadotropin releasing hormone receptor (GnRHR) (Beranova et al. 2001)).

In general, most GPCR disease-causing polymorphisms are either amino acid-altering point mutations or truncations that lead to GPCR misfolding. These structural changes cause a variety of altered pharmacological properties, including reduced ligand binding, loss of intrinsic function, increased intracellular retention, and decreased plasma membrane expression. Well-described examples of polymorphic GPCRs exhibiting such pathopharmacological properties include human V2R and GnRHR (Birnbaumer et al. 1994; Conn and Janovick 2009; Tan et al. 2009; Fortin et al. 2010; Wise et al. 2010); many other similar GPCR polymorphisms have been described (for a complete review, see reference (Conn et al.

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2007)). However, pharmacological dysfunction of polymorphic GPCRs does not always involve receptor misfolding and can be due to other cellular mechanisms.

Several GPCR polymorphic variants have been described that have retained receptor folding and pharmacological function, but only exhibit altered plasma membrane trafficking. For example, a polymorphic form of the human delta opiate receptor (DOR) occurring in the amino terminus retained pharmacological binding properties, but exhibited altered intracellular trafficking, glycosidic processing, and plasma membrane expression (Leskela et al. 2009). Similarly, a naturally occurring third intracellular (iC3) loop mutation in the histamine type 1 (H1) receptor has been shown to alter intracellular trafficking and plasma membrane expression, without abolishing receptor function (Noubade et al. 2008), possibly by altering protein/chaperone interactions. Therefore, altered plasma membrane trafficking alone can be a mechanism of GPCR variant dysfunction.

Naturally occurring GPCR polymorphisms have also been identified in human α2A and α2C adrenergic receptor (AR) subtypes, though linkage to pathophysiological disease states has been inconsistent (Small et al. 2002; Kardia et al. 2008; Rosskopf and Michel 2008; Sehnert et al. 2008). The two most common α2A and α2C AR variants identified occur within the iC3 loop of the receptor, α2A N251K (asparagine to lysine) and α2C Δ322-325 (a four-amino acid deletion) (Small et al. 2000a; Small et al. 2000b). Following heterologous expression in CHO cells, an initial examination of α2A N251K AR pharmacology in membrane fractions demonstrated no differences in ligand binding affinities or basal receptor function. However, enhanced agonist-induced inhibition of adenylyl cyclase and stimulation of mitogen-activated protein (MAP) kinase and inositol phosphate (IP) accumulation were observed (Small et al. 2000a). Similar studies with α2C Δ322-325 ARs revealed a loss of efficacy, demonstrating reduced agonist-promoted coupling to various G_i second messenger systems, including adenylyl cyclase, MAP kinase, and IP production, with no alteration of ligand binding affinities (Small et al. 2000b). Conversely, a more recent examination of α2C Δ322-325 AR pharmacology in intact HEK293 cells demonstrated no such differences in adenylyl cyclase inhibition, utilizing multiple agonists across a range of receptor expression densities. These divergent results may in part be due to differences in cell types utilized, variation in downstream signaling components, or subcellular localization (Montgomery and Bylund 2010).

Other possible mechanisms of receptor dysfunction for these two common α2 AR variants remain unexamined. For example, restricted trafficking may be an important cellular mechanism responsible for regulation of receptor availability and function (Jean-Alphonse and Hanyaloglu 2011). α2A and α2C AR iC3 loop regions have complex overlapping interactions with multiple proteins, including spinophilin, GPCR kinases (GRK), and arrestins, which play a role in intracellular trafficking (Richman et al. 2001; Wang and Limbird 2002; Wang et al. 2004). Despite the extensive biochemical and pharmacological characterization of α2A N251K and α2C Δ322-325 ARs, subcellular localization and possible trafficking defects of these variant forms have not been examined.

A simple comparison of B_{max} values for heterologously expressed receptors cannot discern trafficking alterations, since plasma membrane expression reflects receptor folding kinetics and stability as well as intracellular movement and biochemical processing through organelles (i.e., endoplasmic reticulum (ER) and Golgi). Also, immunofluorescent examination of the intracellular localization of mutant or polymorphic GPCRs cannot adequately discern if receptor trafficking has been altered. For example, a receptor polymorphism that mutates or removes a plasma membrane trafficking motif could lead to ER retention; however, a mutant GPCR that is misfolded and nonfunctional would also be retained in the ER. To truly determine if a GPCR polymorphism affects trafficking only, it must be demonstrated that (1) the polymorphism does not affect GPCR function (i.e., it is not misfolded), (2) the polymorphism leads to alterations in intracellular processing (e.g., glycosylation), and (3) any observed changes in plasma membrane expression are specific and do not simply reflect a change in total cellular expression of the receptor (e.g., both intracellular and plasma membrane expression are not altered concurrently). Therefore, systematic investigation of GPCR trafficking requires a multi-step algorithmic approach to analyze receptor functionality, intracellular localization, glycosidic processing, and finally quantitative examination of plasma membrane and intracellular expression.

Previously, we established a systematic approach for examining intracellular trafficking, processing, and plasma membrane expression of GPCRs for the study of receptor trafficking motifs and accessory proteins, utilizing a combination of biochemical and quantitative fluorescent-activated cell sorting (FACS) methods (Angelotti et al. 2010; Björk et al. 2013; Hurt et al. 2013). Applying this approach to the study of α2A N251K and α2C Δ322-325 ARs, we demonstrate that both variants exhibit similar intracellular glycosidic processing and plasma membrane trafficking compared to their wild-type (WT) counterparts. Thus, any potential pathopharmacological properties of these variant α2 ARs may be due to other intracellular signaling events and not altered cellular processing, receptor function, or plasma membrane expression.

Materials and methods

Materials

Nonidet P-40, Triton X-100, benzamidine, ethylenediaminetetraacetic acid (EDTA), trishydroxymethylaminomethane

(Tris), phenylmethylsulphonyl fluoride (PMSF), pepstatin, aprotinin, cycloheximide, sodium orthovanadate (Na_3VO_4), saponin, and paraformaldehyde (PFA) were obtained from Sigma-Aldrich Corp. Leupeptin was obtained from Roche.

Gene construction and expression

Template complementary DNA (cDNA) of the α2A WT and α2C Δ322-325 genes were obtained from Missouri S&T cDNA Resource Center (Rolla, MO) and reconstituted according to their provided protocol. These templates were mutated to create corresponding α2A N251K or α2C WT using the QuickChange® PCR mutagenesis kit (Stratagene), according to manufacturer's instructions. The α2A WT receptor was modified with a single-base pair substitution at nucleotide 753 to convert a cytosine to guanine (N251K), and a 12-base pair insertion (corresponding to the missing amino acids Gly-Ala-Gly-Pro) was placed into α2C Δ322-325 AR (nucleotide 963-974) to covert it back to α2C WT (Small et al. 2000b). In a similar manner, an hemagglutinin (HA) epitope tag (YPYDVPDYA) was added on the amino terminus of all constructs to facilitate fluorescent antibody labeling as described previously (Hurt et al. 2000). All PCR mutagenesis was performed in the cloning vector pBSKII KS(−) (Stratagene) and bacterial clones were sequenced prior to further experimentation.

For transient expression, cDNAs were inserted into a pCDNA3.1 mammalian expression vector (Invitrogen) and transfected into either HEK293 or Rat1 cells using Effectene (Qiagen), according to the manufacturer's protocol. The parent vector pCDNA3.1 was used as a negative transfection control for FACS experiments. HEK293 and Rat1 cells were cultured at 37 °C and 5 % carbon dioxide with Dulbecco's modified Eagle's media (GIBCO) supplemented with 10 % fetal bovine serum (Gemini Bio Products). HEK293 (ligand binding, glycosidic processing, and quantitative FACS analyses) and Rat1 (immunofluorescence analysis) cells were plated onto 10-cm dishes and transfected at 90 % confluency with 1.5 μg of vector DNA.

Radioligand binding analysis

Total ligand binding was determined by saturation binding with the α2-AR antagonist [^{3}H]RX821002 (GE Healthcare), as described previously (Hurt et al. 2000). Forty-eight hours after transfection, HEK293 cells were washed two times with phosphate-buffered saline (PBS) with 1 mM EDTA and harvested in 5 ml of PBS with 1 mM EDTA and centrifugation at 500×*g* for 5 min. Cell pellets were resuspended in hypotonic lysis buffer (HLB 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) pH 7.4, 1 mM EDTA pH 7.4, 2 μg/ml aprotinin, 1 mM benzamidine, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF, 0.5 mM Na_3VO_4, and 0.03 mM cycloheximide) and homogenized with 30 strokes of a 1-ml Dounce Style Tissue Grinder (Wheaton). Homogenates were centrifuged at 200×*g* for 5 min to clear debris, and cleared homogenate was centrifuged at 16,000×*g* for 45 min to obtain cell membranes. Membrane pellets were resuspended in binding buffer (75 mM Tris, 12.5 mM $MgCl_2$, 1 mM EDTA pH 7.4). Protein concentrations were determined using a DC Protein Assay Kit (Bio-Rad) with BSA as a standard. Total binding was determined using a saturating concentration of [^{3}H] RX821002 (64 nM), with yohimbine (10 μM) added to obtain nonspecific binding. Three separate cDNA transfections were performed, and 20 μg of membrane protein were used per binding reaction in triplicate. Samples were transferred to Whatman GF/C glass filter paper by vacuum filtration using a Brandel M-48 Harvester. Filter paper was put in scintillation tubes and mixed with 5 ml of scintillation fluid overnight. Counts were obtained using a Beckman LS6000IC liquid scintillation counter. Binding data (CPM) was converted to receptor levels (pmol/mg protein), with the mean±standard error of the mean (SEM) calculated for each cDNA construct and plotted using Prism version 6 software (GraphPad, San Diego, CA). Relative expression between all WT and polymorphic α2 ARs was analyzed by one-way ANOVA.

Immunofluorescent microscopy

Immunofluorescent microscopy (IF) was performed as described previously (Hurt et al. 2000). In brief, 24 h post-transfection, transfected Rat1 cells (100–150,000) were seeded on sterile poly-D-lysine-coated glass cover slips. Forty-eight hours post-transfection, cells were rinsed three times with phosphate buffered saline supplemented with calcium and magnesium (PBS-CM). Cells were fixed for 5 min with 4 % PFA at room temperature (RT). Blocking solution (5 % dry milk, 2 % Goat Serum, 50 mM HEPES pH 7.4 in PBS-CM) was used to reduce nonspecific antibody activity. Cells were permeabilized with 0.2 % NP40 in blocking solution. Antibody applications were performed in blocking solution for 1 h at RT using various combinations of antibodies to detect receptors and intracellular organelles. For ER localization, α2 ARs were labeled with mouse monoclonal anti-HA (16B12) (1:500, Covance) and the ER was immunolabeled with rabbit polyclonal anti-calreticulin (1:1,000; Abcam). For co-localization with other organelles, α2 ARs were labeled with rabbit polyclonal antibodies C10 and C4 (generated against the carboxyl termini of α2A and α2C AR, respectively, 1:300) (Daunt et al. 1997). The Golgi apparatus, early endosomes, and lysosomes were labeled with mouse monoclonal antibodies as follows: Golgi, mouse monoclonal anti-GS15 (1:500; BD Transduction Laboratories); early endosomes, mouse monoclonal anti-EEA1 (1:250; BD Transduction Laboratories); and lysosomes, mouse monoclonal anti-Lgp120 (1:1,000; BD Transduction Laboratories). Immunolabeled cells were washed ×3 with PBS-CM at

5 min intervals and blocking solution was reapplied to the cells for 20 min. Secondary antibody (goat anti-mouse Alexa 594 or goat anti-rabbit Alexa Fluor 488 (1:1,000; Invitrogen)) was applied for 1 h in the dark at RT. After secondary labeling, cells were rinsed ×3 with PBS-CM at 5 min intervals in the dark and mounted using Vectashield Hard Set mounting medium H-1500 with DAPI (Vector Laboratories, Inc.). Images were obtained with a Zeiss Axioplan 2 epifluorescence oil immersion microscope under ×630 magnification, and images were acquired by a digital camera (Roper Scientific RTE/CCD) using IPlabs software (Macintosh version). Image brightness and contrast were adjusted using Adobe Photoshop CS3 version 10 (Macintosh version).

Biochemical analysis of cellular processing

HEK293 cells were transfected and membrane pellets were obtained as described above (see "Radioligand binding Analysis"); however, pellets were resuspended in HLB. Membrane preparations (80–100 μg protein) were treated for 4 h with endoglycosidase H_f (Endo H_f) or peptide-*N*-glycosidase (PNGase F) (New England Biolabs) per manufacturer's recommendations. Control and digested preparations were denatured for 10 min at 65 °C after addition of 5X Laemmli buffer and separated on 10 % SDS-PAGE gels. Proteins were transferred to nitrocellulose for immunoblot analysis and labeled with mouse monoclonal anti-HA (16B12 antibody, 1:1,000 dilution, Covance). α2 AR receptors were visualized with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:2,000 dilution, GE Healthcare) and developed using an Amersham ECL kit (GE Healthcare), per manufacturer's instruction.

Quantitative FACS

The proportion of α2 AR constructs expressed at the cell plasma membrane relative to total receptor expression was determined in transiently transfected HEK293 cells using FACS methods, as described previously (Angelotti et al. 2010; Hurt et al. 2013). Forty-eight hours post-transfection, cells were lifted off dishes and resuspended with Hanks buffered saline solution supplemented with 5 mM EDTA, fixed with 2 % PFA for 10 min at RT, and washed three times with PBS at 5 min intervals. For differential analysis of surface and total α2 AR expression, cell preparations were divided into two groups. Surface expression groups (nonpermeabilized) were blocked with PBS supplemented with 2 % fetal bovine serum (FBS) for 45 min prior to fluorescent labeling. Total expression groups were permeabilized with blocking solution containing PBS with 2 % FBS and 0.1 % Triton X-100 for 15 min and then washed once with PBS with 2 % FBS for 30 min. A 1-h incubation with fluorescein isothiocyanate (FITC)-conjugated 16B12 antibody (anti-HA, Covance) in blocking solution (1:500 dilution) was used to label all α2-AR constructs in the two preparations. Similarly treated, untransfected HEK293 cells served as control.

FACS analysis of α2 AR expression was performed using an LSR-I flow cytometer (Becton-Dickinson Bioscience). Fluorescent intensities of 1,000 or more cells were obtained for each construct from permeabilized and nonpermeabilized cell preparations. Less than 1 % of cells were noted to be dead as assessed by propidium iodine staining prior to fixation. After gating to remove untransfected cells from analysis, a minimum of 1,000 cells was used to determine the median fluorescent intensity of each cell preparation. At least three independent experiments were performed to obtain average median fluorescent intensity for each α2 AR construct. The median fluorescent intensities are reported as average±SEM of at least three experiments. The percentage of surface expression of each of the α2 AR constructs was determined by dividing the median surface (nonpermeabilized) fluorescent intensity by the median total (permeabilized) fluorescent intensity. The percentage of intracellular expression was calculated by subtracting the percentage of surface expression from 100 %. Statistical analysis of percent surface and percent intracellular expression was performed using a one-way ANOVA analysis and an unpaired Student's *t* test with Welch's correction (Prism version 6 software, GraphPad, San Diego, CA).

Results

α2A and α2C WT and polymorphic cDNA constructs

In order to utilize the multi-step algorithm described above to examine if common human α2A and α2C AR polymorphisms alter plasma membrane trafficking, cDNAs encoding α2A WT, α2A N251K, α2C WT, and α2C Δ322-325 ARs were created by PCR mutagenesis. Additionally, intracellular immunolocalization and quantitative determination of plasma membrane/intracellular GPCR expression by FACS required an antibody directed against an extracellular receptor epitope. Since no commercially available antisera were available against extracellular epitopes of either WT α2 ARs, an HA epitope was introduced into the extreme amino terminus of all WT and variant forms of α2 ARs. We have previously demonstrated that an introduction of an HA epitope at this site within the highly homologous murine α2A and α2C ARs did not affect pharmacological function or intracellular localization of the receptor (Daunt et al. 1997; Hurt et al. 2000; Angelotti et al. 2010).

Binding analysis of α2A and α2C WT and polymorphic forms

The functionality of heterologously expressed α2A and α2C WT and polymorphic forms has been examined extensively

by agonist/antagonist binding and second messenger signaling analysis, as described above (Small et al. 2000a; Small et al. 2000b; Montgomery and Bylund 2010). In order to ensure that our cDNA constructs encoded properly folded proteins, radioactive ligand binding analysis at a single saturating concentration was performed (Fig. 1). Overall, both WT and polymorphic forms of both α2A and α2C ARs exhibited similar specific ligand binding, respectively, though α2A ARs had higher levels of expression compared to their α2C AR counterparts, consistent with prior studies (Angelotti et al. 2010). There was no statistically significant difference between ligand binding of WT and polymorphic forms of either α2A or α2C ARs.

Immunofluorescent analysis of intracellular localization

Given that all four constructs retained functionality, we next utilized rapid immunofluorescent screening to examine if polymorphic α2A and α2C ARs exhibited any obvious alteration in plasma membrane or intracellular localization/distribution. As shown previously (Hurt et al. 2000; Angelotti et al. 2010), heterologously expressed α2A ARs are found primarily on the plasma membrane, whereas the majority of α2C ARs reside intracellularly, co-localized with endoplasmic reticulum (ER) marker proteins. To quickly examine if the polymorphic forms of either α2 ARs altered intracellular localization, Rat1 cells were transfected with HA-α2 AR WT and variant forms, and the ER, Golgi apparatus, early endosomal, and lysosomal intracellular compartments were identified by wide-field immunofluorescent microscopy, following antibody labeling of α2 ARs and specific organelle marker proteins. Rat1 cells were chosen over HEK293 cells for immunofluorescent screening due to their larger cytoplasmic/nuclear ratio, which allowed for easier identification of intracellular organelles. Antisera against the following marker proteins were utilized to identify the various compartments: ER (calreticulin), Golgi (GS15), early endosome (EEA1), and lysosome (Lgp120).

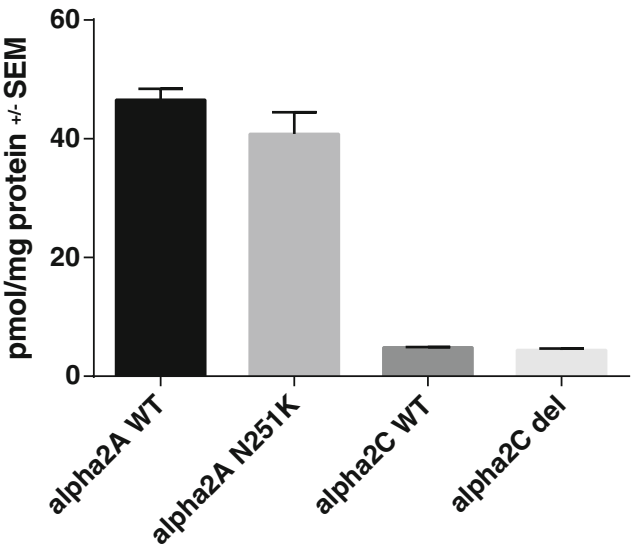

Fig. 1 Radioligand binding properties of WT and polymorphic α2 ARs. HEK293 cells were transfected with either WT or polymorphic HA-α2A or -α2C ARs. Forty-eight hours post-transfection, crude membranes were isolated and subjected to radioligand binding with a saturating concentration of [^{3}H]RX821002 (64 nM), as described previously (Angelotti et al. 2010). Total and nonspecific bindings were measured in triplicate, and specific binding is shown in picomoles per milligram protein. Values shown represent the mean±SEM of three independent experiments, analyzed by one-way ANOVA. No statistically significant difference in total expression levels was noted between WT and polymorphic forms of either α2A or α2C ARs

As expected, WT α2A and α2C ARs were expressed primarily at the plasma membrane and ER, respectively. Our analysis of α2A N251K AR localization showed no noticeable variation from WT α2A ARs (Fig. 2). Both were clearly expressed at the plasma membrane as indicated by their membrane demarcation along the cell body, with some intracellular retention that co-localized to the ER, as delineated by calreticulin staining. Neither showed significant co-localization with early endosomal or lysosomal compartments, though some co-localization within Golgi was noted, consistent with previous experiments (Daunt et al. 1997; Hurt et al. 2000). Overall, the intracellular localization pattern did not appear to be altered. Similarly, α2C Δ322-325 ARs demonstrated similar localization as their WT counterpart, with the majority of the expressed protein being found in the ER (co-localized with calreticulin) and a small amount localized to the plasma membrane (Fig. 3). Again, no apparent alteration was observed with respect to other intracellular compartments examined (e.g., Golgi, early endosome, lysosome). Since no obvious alteration in localization was noted for either WT or polymorphic forms of α2A and α2C ARs, further detailed confocal microscopic analysis was not performed.

Glycosidic analysis of α2 AR processing

Rapid immunofluorescent screening of WT and polymorphic α2 ARs did not discern any obvious alterations in plasma membrane or intracellular localization, suggesting that these α2 AR polymorphisms did not grossly alter intracellular trafficking. However, immunofluorescent microscopy is not a sensitive method for discerning such changes. As GPCRs traffic through the secretory pathway from ER to Golgi to plasma membrane, they undergo glycosidic processing and maturation. If a GPCR polymorphism altered intracellular trafficking, then it should also affect glycosidic processing of the receptor protein, as was observed with the DOR polymorphism (Leskela et al. 2009). Similar changes in glycosidic processing have been seen following mutation of a known GPCR trafficking motif within α2C ARs (Angelotti et al. 2010).

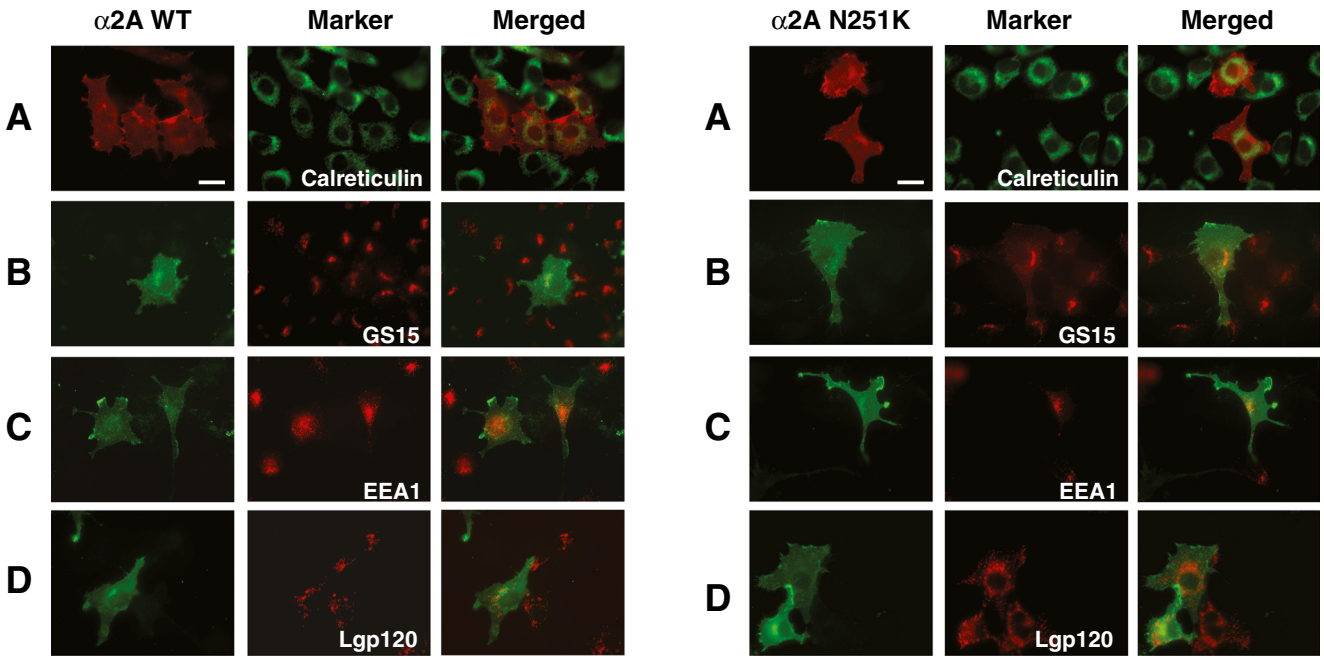

Fig. 2 Immunofluorescent analysis of α2A WT and N251K AR expression. Cellular localization of HA-α2A WT (*left*) and N251K (*right*) ARs were determined in transiently transfected Rat1 cells. Forty-eight hours after transfection, cells were fixed and immunolabeled with anti-HA antibody 16B12 (*A*) or anti-α2A AR antibody C10 (*B*, *C*, *D*) to localize HA-α2A ARs. ER, Golgi compartment, early endosomes, and lysosomes were immunolabeled with antibodies against *calreticulin* (*A*), *GS15* (*B*), *EEA1* (*C*), and *Lgp120* (*D*), respectively. Merged images are shown. As described previously, both HA-α2A WT and N251K ARs demonstrated similar strong expression at the plasma membrane, with minimal intracellular localization noted (Angelotti et al. 2010). The staining for both WT and polymorphic HA-α2A AR forms did not reveal an altered localization with respect to any organelle marker protein tested (calreticulin, GS15, EEA1, or Lgp120). Representative of three separate transfections. *Scale bars* 10 μm

The glycosylation state of a receptor can be assessed by digestion of GPCR-containing membranes with endoglycosidase H (Endo H) and peptide:*N*-glycosidase F (PNGase F). Endo H cleaves asparagine-linked high-mannose containing glycans from glycoproteins that have not trafficked from the ER to the *cis*-medial Golgi apparatus. Such glycoproteins reside within the ER as immature proteins. PNGase F cleaves all asparagine-linked glycans regardless of their status as immature or mature glycoproteins, or their location. By digesting expressed GPCR glycoproteins with endoglycosidases, it is possible to analyze the relative content of immature and mature forms of WT α2 ARs and compare them to their polymorphic counterparts, in order to determine if the α2A N251K and α2C Δ322-325 ARs demonstrate altered intracellular processing that cannot be discerned by rapid immunofluorescent screening.

HEK293 cells were transfected with HA-α2A WT, -α2A N251K, -α2C WT, or -α2C Δ322-325 ARs and membrane fractions were analyzed by endoglycosidase analysis. α2 AR WT and variant forms demonstrated no alteration in glycosylation patterns or relative amounts of immature and mature glycosylated receptor forms (Fig. 4). Both α2A WT and N251K ARs were predominantly found with mature glycosylation (55–70 kDa, Endo H insensitive) and a smaller amount of immature glycosylated form of the receptor (45–50 kDa, Endo H sensitive), as described previously (Angelotti et al. 2010). Both forms were sensitive to PNGase F digestion. Similarly, α2C WT and Δ322-325 ARs also were found with similarly sized mature and immature glycosylated forms; however, the immature (Endo H sensitive) form predominated, consistent with its ER localization. Therefore, expressed variant forms of α2 ARs did not alter glycosidic processing of either receptor as it trafficked through the secretory pathway.

Quantitative FACS analysis of plasma membrane expression

Qualitative immunofluorescent microscopy can discern the cellular localization of a GPCR; however, it is not sensitive enough to measure changes in surface (plasma membrane) expression. Glycosidic analysis can determine if intracellular processing has been altered by a mutation or polymorphism. However, if the polymorphism merely enhanced or delayed trafficking, it would not necessarily alter the glycosylation patterns seen, but could affect the level of expression found on the plasma membrane. FACS analysis is a unique assay that can detect and quantify individual cells based upon immunofluorescent labeling and thus allow for relative measurements of receptor expression. Unlike ligand binding, FACS can be used to quantify the relative plasma membrane and total cell receptor levels for a GPCR. From these measurements, the precent surface and precent intracellular expression can be calculated in order to determine with more certainty if a

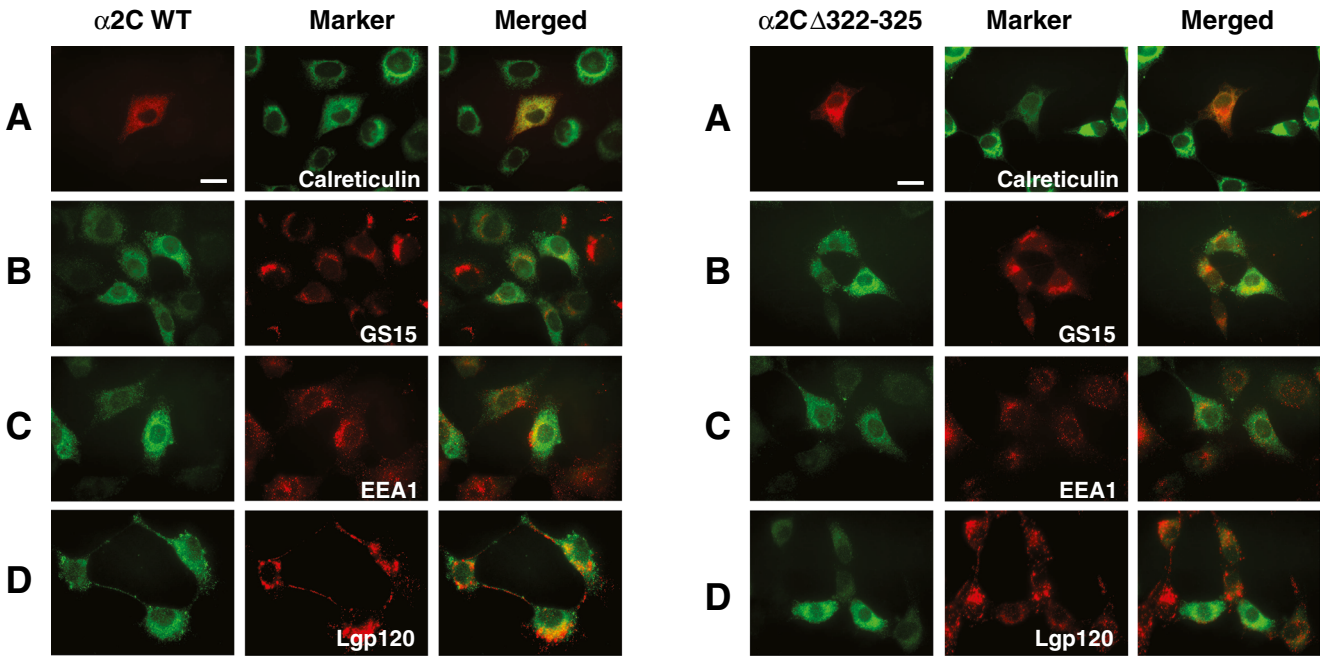

Fig. 3 Immunofluorescent analysis of α2C WT and Δ322-325 AR expression. Cellular localization of HA-α2C WT (*left*) and Δ322-325 (*right*) ARs were determined in transiently transfected Rat1 cells. Forty-eight hours after transfection, cells were fixed and immunolabeled with anti-HA antibody 16B12 (*A*) or anti-α2C AR antibody C4 (*B*, *C*, *D*) to localize HA-α2C ARs. ER, Golgi compartment, early endosomes, and lysosomes were immunolabeled with antibodies against *calreticulin* (*A*), *GS15* (*B*), *EEA1* (*C*), and *Lgp120* (*D*), respectively. Merged images are shown. Both HA-α2C WT and Δ322-325 ARs demonstrated similar strong intracellular expression, with minimal plasma membrane expression. As described previously (Angelotti et al. 2010), the intracellular staining for both HA-α2C AR forms was similar to the ER marker calreticulin, but not other organelle marker proteins (GS15, EEA1, or Lgp120); HA-α2C WT and Δ322-325 ARs exhibited similar intracellular localization patterns. Representative of three separate transfections. *Scale bars* 10 μm

polymorphism (or mutation) affected trafficking and thus plasma membrane expression (Angelotti et al. 2010; Hurt et al. 2013). Therefore, quantitative FACS allows for simultaneous measurement of relative plasma membrane and intracellular GPCR expression and thus could determine if either α2A N251K or α2C Δ322-325 ARs had altered plasma membrane expression or trafficking compared to WT receptors.

HEK293 cells were transfected with HA-α2A WT, -α2A N251K, -α2C WT, or -α2C Δ322-325 ARs and α2 AR expression was quantified with FACS by labeling with a FITC-conjugated anti-HA antibody. Transfected cell populations were divided, and surface (plasma membrane) and total (plasma membrane+intracellular) receptor levels were measured under nonpermeabilized and permeabilized conditions, respectively. Therefore, for a given FACS histogram, the fluorescence intensity distribution represents either total receptor (permeabilized) or only plasma membrane (nonpermeabilized) receptor expression. The variability in fluorescence intensity seen within a histogram represents the heterogeneity of α2 AR expression levels measured in single cells following transfection (Björk et al. 2005). For α2C ARs, we observed more variability in plasma membrane expression compared to α2A ARs. The finding of a more heterogeneous α2C AR plasma membrane expression is consistent with our previous immunofluorescent microscopic analysis (Björk et al. 2013).

As can be seen in a representative FACS histograms (Fig. 5), α2A AR-expressing cells exhibit a higher median fluorescent intensity in nonpermeabilized cells (surface expression), compared to α2C ARs, consistent with our prior data demonstrating a higher level of α2A AR plasma membrane expression in HEK293A cells (Angelotti et al. 2010). Following permeabilization (total expression), α2A and α2C AR expressing cells demonstrate a shift towards higher median fluorescent intensities (Fig. 5 and Table 1). This effect is more pronounced for α2C ARs, since they have a larger intracellular pool of receptor due to ER retention, which is detected only under permeabilized conditions (Angelotti et al. 2010). Similar FACS histograms for permeabilized and nonpermeabilized cells were observed following expression of α2A N251K and α2C Δ322-325 ARs (Fig. 5). Note that total median fluorescence intensities for α2A and α2C WT and polymorphic ARs (Supplemental Fig. S1) correlated with whole membrane receptor expression measured by ligand binding (Fig. 1).

Because WT and polymorphic α2A and α2C ARs express at different levels, a direct comparison of surface and total median fluorescent intensities between WT and polymorphic forms would not be appropriate to detect differences in plasma membrane trafficking. More importantly, it is necessary to compare the relative amounts of plasma membrane and

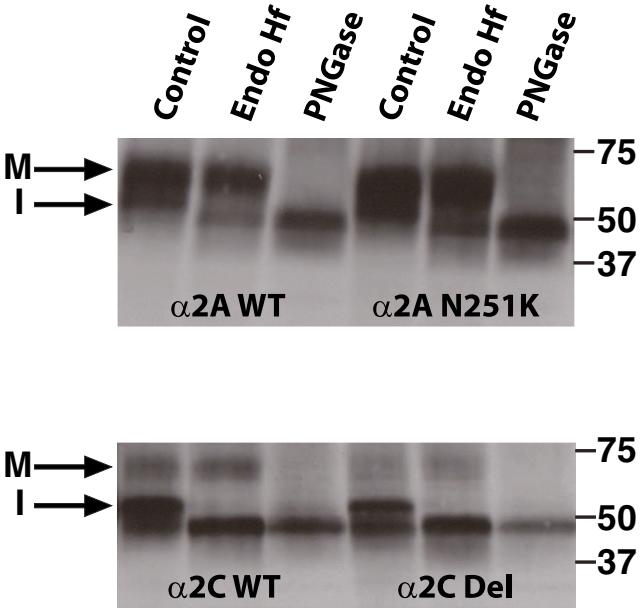

Fig. 4 Glycosidic processing analysis of WT and polymorphic α2 ARs. HEK293 cells were transfected with either WT or polymorphic HA-α2A or -α2C ARs. Forty-eight hours post-transfection, crude membranes were isolated and subjected to endoglycosidase digestion with no enzyme (*control*), endoglycosidase H_f (*Endo Hf*), or PNGase F (*PNGase*). Due to loss of signal during enzymatic digestion, 100 μg of protein was digested and loaded in each lane. Mature (*M*) and immature (*I*) glycosylated forms are indicated. Endo H cleaves only immature glycosylated forms, whereas PNGase cleaves all glycosylated forms. Molecular weight markers (kDa) are shown on the *right*. Note that HA-α2A WT and -α2A N251K ARs exhibit mostly mature (Endo H insensitive), whereas HA-α2C WT and -α2C Δ322-325 ARs show mostly immature (Endo H sensitive), glycosylation patterns, correlating with their predominant plasma membrane and intracellular localizations, respectively. The glycosylation pattern of neither polymorphic HA-α2 AR differed significantly with respect to its WT counterpart. Since α2 ARs are highly susceptible to degradation and aggregation, which can vary between experiments, attempts at quantification of any form of the receptor would not be reproducible and were not performed. Representative of three experiments

intracellular receptor expression in order to demonstrate possible alterations in plasma membrane trafficking. Since it is not possible to directly measure intracellular receptor expression using this methodology, intracellular receptor expression is calculated as the normalized difference between total and plasma membrane receptor expression as described above (see "Methods") (Hurt et al. 2013). Therefore, we calculated the percentage of plasma membrane (surface) and intracellular expression for each receptor from the median total and surface fluorescent intensities in order to normalize the four receptor populations (Table 1). FACS analysis revealed no statistical difference between percent surface expression of α2A WT and N251K (60.5±5.7 vs. 71.6±6.0, respectively) nor α2C WT and α2C Δ322-325 ARs (45.8±7.9 vs. 40.9±9.1), demonstrating that neither polymorphic form altered plasma membrane expression.

Discussion

Within the sympathetic nervous system (SNS), α2A and α2C ARs serve as autoreceptors to limit release of norepinephrine and epinephrine from sympathetic nerve terminals and adrenal chromaffin cells at rest (Brede et al. 2003; Hurt and Angelotti 2007). Initial pharmacological analysis of α2 AR variants suggested that α2A N251K may have a gain-of-function phenotype, whereas α2C Δ322-325 may have a loss-of-function phenotype (Small et al. 2000a; Small et al. 2000b). It was suggested that altered receptor function may be relevant to cardiovascular diseases (Small and Liggett 2001). However, linkage of polymorphic α2A and α2C ARs to pathophysiological mechanisms, such as congestive heart failure and response to treatment, has been examined with inconsistent results (Small et al. 2002; Kardia et al. 2008; Rosskopf and Michel 2008; Sehnert et al. 2008; Bristow et al. 2010). The lack of clear genetic linkages may be due to the finding that both variant forms of α2 ARs exist as multiple haplotypes, and expression of specific haplotypes may be more important determinants of cardiovascular disease risk (Small et al. 2004; Small et al. 2006). Despite the lack of a clear linkage to cardiovascular disease, it has been demonstrated that human α2C Δ322-325 AR homozygotic volunteers have the pathophysiological trait of increased sympathoneural drive (Neumeister et al. 2005).

Subsequent pharmacological analysis of α2C Δ322-325 ARs using intact HEK293 cells suggested no loss-of-function phenotype, suggesting that if this variant form did have a linkage to pathophysiological conditions (such as increased sympathoneural drive), other cellular mechanisms of receptor dysfunction must play a role. Many well-characterized polymorphic forms of other GPCRs (e.g., V2R, GnRHR, rhodopsin) have revealed that altered plasma membrane expression or trafficking can account for dysfunction of the encoded receptor, leading to pharmacological and physiological dysfunction and thus disease (Conn et al. 2007). In most examples, the identified GPCR polymorphism led to amino acid alterations or truncations, which led to receptor misfolding, intracellular retention, and thus decreased or absent plasma membrane expression. Some misfolded receptors could be rescued by addition of a pharmacological chaperone ("pharmacoperone") to enhance proper folding and ultimately lead to proper membrane expression and pharmacological function (Conn and Ulloa-Aguirre 2010). However, other GPCR polymorphisms have been shown to alter intracellular trafficking and/or plasma membrane expression without affecting receptor function, such as certain naturally occurring DOR and H1 receptor polymorphisms (Noubade et al. 2008; Leskela et al. 2009). Thus, intracellular misrouting of receptors may be responsible for dysfunction of some variant GPCRs.

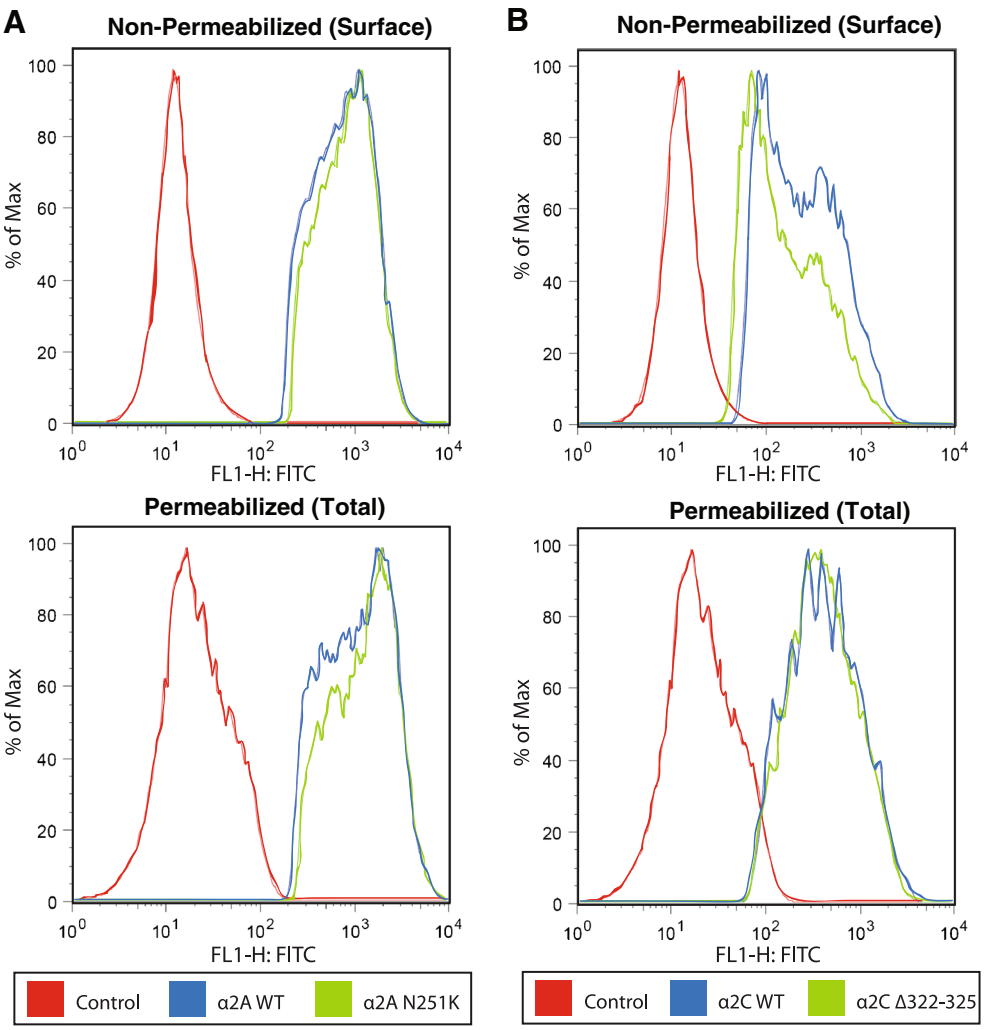

Fig. 5 Representative FACS histograms. HEK293 cells were co-transfected with WT and polymorphic HA-α2A or -α2C AR cDNAs. Forty-eight hours post-transfection, relative expression levels of each receptor were determined under nonpermeabilized (surface) and permeabilized (total=surface+intracellular) conditions by using a FACS assay with a FITC-conjugated anti-HA antibody. A minimum of 1,000 cells was analyzed for each transfection. **a** Representative HA-α2A WT and N251K AR FACS fluorescence distributions under nonpermeabilized (*top*) and permeabilized (*bottom*) conditions (control=untransfected). **b** Representative HA-α2C WT and Δ322-325 AR FACS fluorescence distributions under nonpermeabilized (*top*) and permeabilized (*bottom*) conditions (control=untransfected). Note the shift to higher median fluorescence upon permeabilization, which is greater for HA-α2C vs. -α2A ARs, due to the larger pool of intracellular HA-α2C ARs (Angelotti et al. 2010)

Studies of α2A and α2C ARs have revealed that they exhibit a complex regulation of intracellular trafficking. It has been demonstrated that α2A and α2C ARs localize to different sites within cultured sympathetic ganglion neurons (SGN) (extra-synaptic and pre-synaptic, respectively) (Brum et al. 2006). Additionally, they exhibit altered intracellular localization when heterologously expressed in various cell lines (Daunt et al. 1997; Hurt et al. 2000). Specifically, α2C ARs exhibit ER retention when expressed in HEK293 cells, but not PC12 cells (a neuronal cell line), which is in part due to an extracellular amino terminal trafficking domain (Angelotti et al. 2010). It has been suggested that WT α2C ARs retained in the ER may be physiologically and pharmacologically functional, since they have been shown to be ligand binding competent (Hurt et al. 2000). Chaperones and other accessory proteins have been shown to alter α2C AR intracellular trafficking or glycosidic processing. For example, α2C ARs exhibit temperature-sensitive trafficking that can be modulated by HSP90 (Filipeanu et al. 2011). Additionally, we have recently shown that members of the receptor expression enhancing protein (REEP) family can enhance the expression of and interact with a minimally glycosylated form of α2C ARs, but not α2A ARs (Björk et al. 2013).

Table 1 FACS determination of the relative surface and intracellular expression of WT and polymorphic α2 ARs

Receptor	Median surface fluorescence	Median total fluorescence	Percent surface expression	Percent intracellular expression
α2A WT	653±154	1,047±177	60.5±5.7	39.5±5.7
α2A N251K	695±129	993±230	71.6±6.0	28.4±6.0
α2C WT	180±40	391±19	45.8±7.9	54.2±7.9
α2C Δ322-325	144±15	362±44	40.9±9.1	59.1±9.1
Untransfected	12±1	16±2	n/d	n/d

Fluorescence intensity represented as arbitrary units. Values given are the average±SEM (n=3)

n/d not determined

Analysis of GPCR trafficking involves more than simply measuring the amount of expressed receptor on the cell surface. If a GPCR polymorphism led to a generalized increase or decrease in receptor synthesis or stability, it would increase or decrease both plasma membrane and intracellular expression levels simultaneously. Therefore, if only plasma membrane receptors were measured, a polymorphic GPCR would appear to have an altered plasma membrane expression, which in reality was a change in total receptor expression. Therefore, a simple comparison of B_{max} values in whole membrane preparations following heterologous expression of WT and variant α2 ARs would not accurately assess plasma membrane trafficking changes, since plasma membrane and intracellular receptor expression were not independently measured. Alternatively, whole cell binding analysis with membrane-impermeant ligands would only measure plasma membrane (and not intracellular) receptor levels, thus preventing a comparison of the relative amounts of receptor trafficking to the plasma membrane compared to intracellular receptor compartments. To overcome these limitations of binding assays, we used a quantitative FACS analysis that we developed for the examination of GPCR trafficking motifs and accessory proteins, to determine if altered receptor trafficking may be a possible mechanism of dysfunction for common α2A and α2C variant forms.

As described above, a complete analysis of polymorphic GPCR trafficking should include analyses of receptor functionality, intracellular localization, glycosidic processing, and quantitative measurement of plasma membrane and intracellular expression. Following a demonstration of receptor function and proper folding by radioligand binding, a rapid immunofluorescent microscopic screening revealed that WT and variant forms of α2A and α2C ARs co-localized to similar cellular regions, the plasma membrane and ER, respectively. Additionally, they did not exhibit altered intracellular localization with respect to the ER, Golgi process, early endosomes, or lysosomes. Furthermore, endoglycosidase analysis revealed no differences in intracellular glycosidic processing or the relative amounts of immature and mature glycosylated forms of either WT or variant forms of α2 ARs.

Most importantly, quantitative FACS analysis demonstrated that α2A WT and N251K ARs had similar amounts of plasma membrane expression, whereas α2C WT and α2C Δ322-325 ARs had similar levels of intracellular expression. Therefore, these common α2 AR variant forms do not appear to alter plasma membrane receptor trafficking, as had been seen for DOR and H1 receptor variants. However, we did observe that human WT α2A and α2C ARs exhibited different levels of surface expression (60.5 and 45.8 %, respectively) compared to their murine counterparts (92 and 25 %, respectively) (Angelotti et al. 2010). The differences seen between these two studies may represent a species effect, reflecting structural/folding/stability dissimilarities due to other amino acid changes between species. By applying a complete algorithmic approach to assess receptor trafficking, it can be concluded that neither polymorphic α2 AR form altered plasma membrane expression. Since the pharmacology and trafficking of these polymorphic α2 ARs are not altered, it is possible that these variant forms may affect other intracellular signaling cascades that involve the iC3 loop, such as spinophilin, GRKs, and arrestins (Richman et al. 2001; Wang et al. 2004). It has been demonstrated that agonist-stimulated interactions between these proteins and α2 ARs are important determinants of endosomal receptor recycling and downregulation (Wang and Limbird 2002). Though some pathophysiological effects have been described (e.g., α2C Δ322-325 AR and increased sympathoneural drive), pathopharmacological properties of α2 AR variant forms remain to be determined and may involve other intracellular modulators.

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