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The Role of a Conserved Inter-Transmembrane Domain Interface in Regulating α_{2a}-Adrenergic Receptor Conformational Stability and Cell-Surface Turnover

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ABSTRACT

Functional and structural data from G protein-coupled receptors (GPCR) predict that transmembrane-domain (TM)2 is adjacent to TM7 within the GPCR structure, and that within this interface a conserved aspartate in TM2 and a conserved asparagine in TM7 exist in close proximity. Mutation at this D79(TM2)-N422(TM7) interface in the α_{2A}-adrenergic receptor (α_{2A}AR) affects not only receptor activation but also cell-surface residence time and conformational stability. Mutation at TM2(D79N) reduces allosteric modulation by Na^+ and receptor activation more dramatically than affecting cell-surface receptor turnover and conformational stability, whereas mutation at TM7(N422D) creates profound conformational instability and more rapid degradation of receptor from the surface of cells despite receptor activation and allosteric modulation properties that mirror a wild-type receptor. Double mutation of TM2 and 7(D79N/N422D) reveals phenotypes for receptor activation and conformational stability intermediate between the wild-type and singly mutated α_{2A}AR. Additionally, the structural placement of a negative charge at this TM2/TM7 interface is necessary but not sufficient for receptor structural stability, because mislocalization of the negative charge in either the D79Eα_{2A}AR (which extends the charge out one methylene group) or the D79N/N422Dα_{2A}AR (placing the charge in TM7 instead of TM2) results in conformational lability in detergent solution and more rapid cell-surface receptor clearance. These studies suggest that this interface is important in regulating receptor cell-surface residence time and conformational stability in addition to its previously recognized role in receptor activation.

The α_2-adrenergic receptors (α_2AR) are members of the superfamily of seven transmembrane spanning G protein-coupled receptors (GPCR) and are coupled via Gi/Go proteins to multiple effectors in native cells such as inhibition of adenylyl cyclase (Limbird, 1988), suppression of voltage-gated Ca^{2+} currents (Horn and McAffee, 1980), activation of receptor operated K^+ currents (Morita and North, 1981), and stimulation of the mitogen-activated protein (MAP) kinase cascade (Richman and Regan, 1998).

Cell-surface receptor expression is necessary for extracellular to intracellular communication mediated by circulating hormones. Mechanisms regulating the long-term cell-surface residence time of GPCRs are poorly understood. For the α_{2A}AR, the presence of the third intracellular loop (Edwards and Limbird, 1999) as well as conformational stability (Wilson and Limbird, 2000) contributes to maintaining long-term (approximately 12–14 hours) cell-surface receptor residence time, albeit through differing mechanisms. Although conformational stability likely contributes to maintaining or permitting cell-surface receptor expression, sites critical for maintaining conformational stability within GPCR structure are only beginning to be elucidated. One such site apparently critical for regulating α_{2A}AR conformational stability is the highly conserved D79 residue, located in a topologically homologous position in all amine-binding GPCR in TM2 (Wilson and Limbird, 2000).

The three-dimensional structural characterization of bacteriorhodopsin and subsequently bovine rhodopsin (Unger et al., 1997; Palczewski et al., 2000), in conjunction with intentional mutagenesis strategies coupled with functional studies (Mizobe et al., 1996; Gether and Kobilka, 1998), has led to a proposed arrangement of the seven helices of GPCR within the bilayer. For amine-binding GPCR, a hydrogen bonding network consisting of at least a conserved aspartate in transmembrane-domain 2 (TM2) and an asparagine in TM7 is

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ABBREVIATIONS: α_2AR, α_2-adrenergic receptor; GPCR, G protein-coupled receptors(s); TM, transmembrane domain; MAP, mitogen activated protein; GnRH; gonadotropin-releasing hormone; HA, hemagglutinin; CHO, Chinese hamster ovary; EcR, ecdysone receptor-expressing; HEK, human embryonic kidney; FCS, fetal calf serum; PMSF, phenylmethylsulfonyl fluoride; [^{125}I]PIC, *para*-[^{125}I]iodoclonidine; GppNHp, 5′-guanylylimidodiphosphate; WT, wild-type; PAGE, polyacrylamide gel electrophoresis; DβM/CHS, dodecyl-β-D-maltoside/cholesteryl hemisuccinate; CAPS, 3-(cyclohexylamino)propanesulfonic acid; $t_{1/2}$, half-life.

postulated to serve as a critical link between agonist occupancy and G protein activation. An exception of nature is the gonadotropin releasing hormone (GnRH) receptor, where these two residues are interchanged such that there exists an asparagine in TM2 and an aspartate in TM7 (Zhou et al., 1994). Exchanging these residues between TM2 and TM7 in the GnRH receptor (creating the N87D/D318N GnRH-R) results in a receptor structure that retains high affinity agonist binding and coupling to G proteins, albeit not to the same extent as the wild-type receptor. A single mutation, N87D in TM2 of the GnRH receptor, however, leads to loss of G protein coupling, presumably because of disruption of this hydrogen bonding network, which serves to regulate receptor activation (Zhou et al., 1994). For the GnRH receptor, this interface can also affect receptor expression in transfected cells (Flanagan et al., 1999). An analogous interaction between an aspartate in TM2 and an asparagine in TM7 has been demonstrated for the $5HT_{2A}$-receptor (Zhou et al., 1994) and for the μ-opioid receptor (Xu et al., 1999). In contrast, complementary TM2/TM7 interface mutation in the cannabinoid CB1 receptor does not restore receptor-mediated potentiation of inwardly rectifying potassium channel current or receptor internalization (Roche et al., 1999), indicating greater complexity of this predicted charge relay system or lack of generality among all GPCR subclasses. Additionally, the recent crystal structure of rhodopsin reveals that Asp83 (TM2) and Asn302 (TM7) are probably too far apart for a direct hydrogen bonding interaction although they may be bridged by a water molecule, revealing a proximity between TM2 and TM7 in GPCR structure (Palczewski et al., 2000).

With regards to the α_{2A}AR, previous studies have implicated several consequences of mutating this highly conserved aspartate (D79) in TM2 including: 1) changes in allosteric modulation of receptor conformation by monovalent cations, such as Na^+ (Horstman et al., 1990; Neve et al., 1991; Kong et al., 1993; Tian and Deth, 1993), 2) altered cell surface residence time of the α_{2A}AR, and 3) diminished conformational/structural stability of the receptor, manifest by loss of functional binding capacity in detergent preparations without parallel changes in receptor protein (Wilson and Limbird, 2000). The present studies explored the role of the conserved TM2(D79)/TM7(N422) interface in the α_{2A}AR and evaluated what role this interface plays in regulating conformational stability, cell-surface residence time and multiple functional properties of the α_{2A}AR.

Materials and Methods

Molecular Modeling. To generate a schematic of the proposed proximity of D79 in TM2 and N422 in TM7 of the α_{2A}AR, molecular modeling was used to generate a simplified model of this receptor. De novo modeling of the α_{2A}AR was performed via e-mail using SWISS MODEL in the GPCR mode (*http://www.expasy.ch/swissmod/SWISS-MODEL.html*). Briefly, the predicted transmembrane domain spans for the α_{2A}AR were entered into the program using the human β_2-AR as the template for modeling. SWISS MODEL then generated models with ProModII and conducted energy minimization with Gromos 96 (Peitsch et al., 1996; Ghex et al., 1999). Swiss-PdbViewer was then used to analyze and visualize the results (Fig. 1).

DNA Reagents. Porcine hemagglutinin (HA)-tagged wild-type, D79N, D79E, and D79Q α_{2A}AR have been described previously (Ceresa and Limbird, 1994). The N422D mutation was engineered simultaneously into the wild-type and D79N backbones in the pCMV4 mammalian expression vector using QuickChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA). Two complementary oligonucleotides generating the N422D mutation with an additional *Bam*HI site to facilitate screening (antisense, 5′-GTAGATAACCGGATCCAGCGAGCTGTTGCAGTAG-3′; sense, 5′-CTACTGCAACAGCTCGCTGGATCCGGTTATCTAC-3′) were used in polymerase chain reactions according to the manufacturers instructions, except that 10% dimethyl sulfoxide was used in the reaction to facilitate extension of the template. Colonies were screened via *Bam*HI digest of minipreps and the entire coding region of the N422D and D79N/N422D mutants was then sequenced with ^{33}P-thermosequenase-cycle sequencing.

Cell Culture and Transfection. EcR-CHO (Chinese hamster ovary cells engineered for the ecdysone-inducible expression system obtained from Invitrogen, (Carlsbad, CA) cells were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 100 U/ml penicillin G sodium with 100 μg/ml streptomycin sulfate (pen-strep). Human embryonic kidney (HEK) 293 cells were maintained in minimal essential medium supplemented with 10% FCS plus pen-strep. EcR-CHO and HEK293 cells were plated the day before transfection at a density of 2×10^6 cells per well of a six-well plate or 35-mm dish. Cells were transiently transfected with the use of FuGENE-6 (Boehringer Mannheim) according to the manufacturer's instructions. Cells were assayed approximately 48 h after transfection.

Guanine Nucleotide Sensitivity of Radiolabeled Agonist Binding as a Measure of α_{2A}AR-G protein Coupling. To evaluate the ability of Gpp(NH)p, a hydrolysis-resistant GTP analog, to modulate radiolabeled agonist binding, membranes from HEK 293 cells transiently expressing receptor were lysed in hypotonic lysis buffer [15 mM HEPES, 5 mM, EDTA, 5 mM EGTA, pH 8.0 (with the addition of 10 U/ml aprotinin and 0.1 mM PMSF)]. Cells were scraped on ice into ice-cold hypotonic lysis buffer and passaged five times through a 25-gauge needle on ice and centrifuged for 15 min at 40,000*g*, followed by resuspension in lysis buffer and recentrifugation. Membranes were then resuspended in 50 mM Tris-HCl, 10 mM $MgCl_2$, and 5 mM EGTA, pH 8.0. Incubations (100 μl) containing membranes, 0.9 nM *para*-[^{125}I]iodoclonidine ([^{125}I]PIC) agonist radioligand (approximately 160,000 cpm/100-μl incubation) and none (control) or increasing concentrations of Gpp(NH)p were incubated for 30 min at 25°C. Reactions were terminated via vacuum filtration using a Brandel Cell Harvester and ice-cold 25 mM glycylglycine, pH 7.6. Filters were then counted in a Beckman 4000 gamma counter (Beckman Instruments, Palo Alto, CA).

Assessment of Allosteric Modulation of α_{2A}AR Conformation by Na^+. Epinephrine was evaluated as a competitor for [^{3}H]RX821002, a radiolabeled antagonist, in the presence or absence of Na^+ (Horstman et al., 1990; Ceresa and Limbird, 1994; Lakhlani et al., 1997). Transiently transfected HEK 293 cells expressing receptor were lysed, centrifuged, resuspended, and recentrifuged as outlined above. Membranes were then resuspended in a small volume of lysis buffer using a 25-gauge needle. A small volume of membranes was then added to a binding reaction consisting of 25 mM HEPES, 40 mM glycylglycine, 100 mM NaCl or *N*-methyl D-glucamine chloride, and 5 mM EDTA, pH 8.0 (final volume, 250 μl) and [^{3}H]RX821002 with none (control) or various concentrations of epinephrine (competitor). Reactions proceeded at 25°C for 30 min and were terminated as above. Filters were counted in Aquasol (Packard, Meriden, CT) in a Packard 1600 TR scintillation counter.

Mitogen-Activated Protein Kinase Assay. The ability of receptor to activate MAP kinase was evaluated in HEK 293 cells transiently expressing α_{2A}AR, WT, or mutant structures. Cells were serum starved overnight starting 24 after transfection. The following day, response to various concentrations of epinephrine (in serum-free medium) was examined for 2 min using one transfected 35-mm dish per condition. The agonist-containing medium was then aspirated and the incubation was terminated by scraping cells into 100

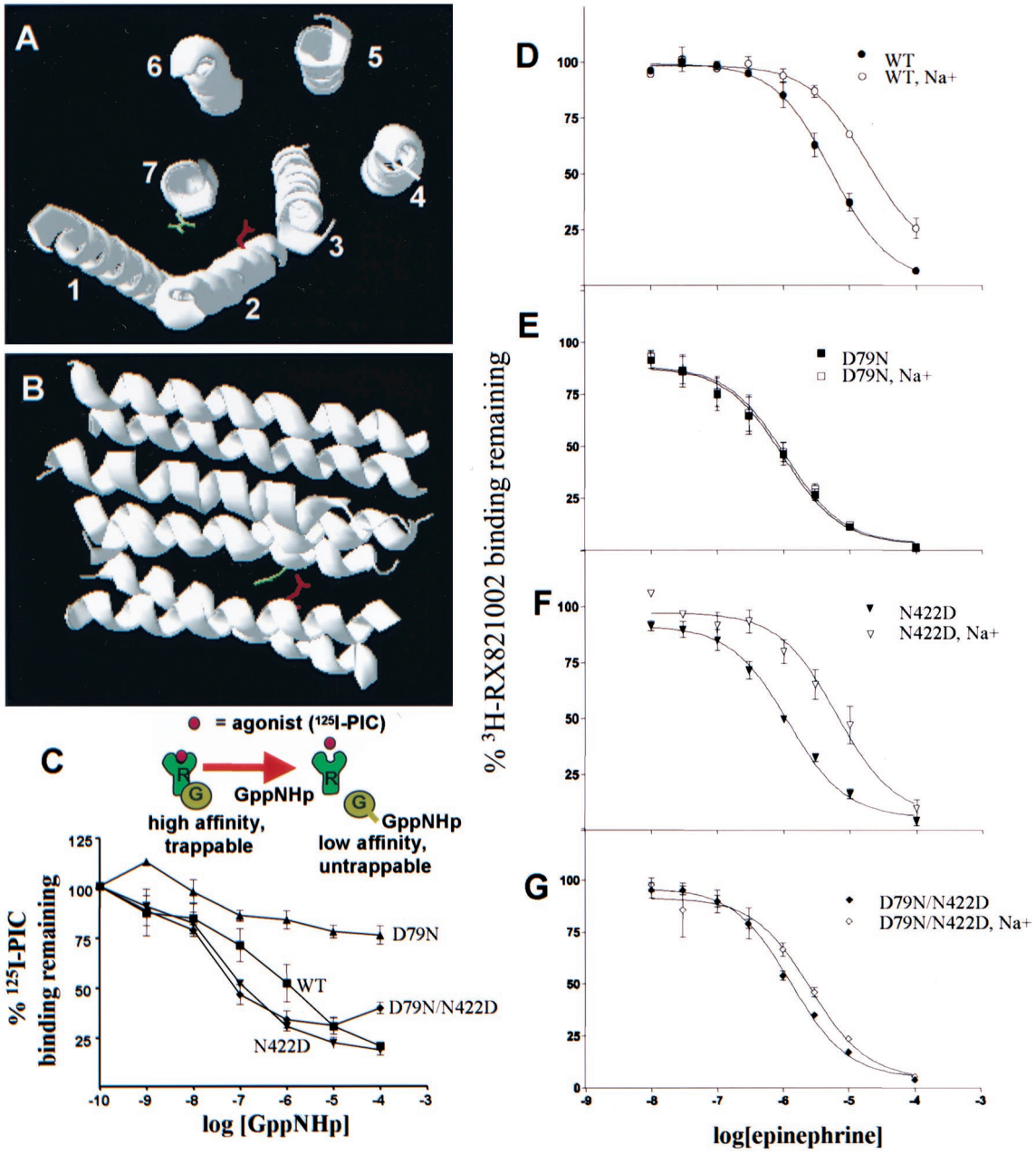

Fig. 1. Structural and functional properties of α_{2A}-adrenergic receptors. A and B, schematic model of the α2AAR showing the predicted proximity of D79(TM2) and N422(TM7). The top (1A) and side (1B) views show residues D79 in red and N422 in green. These models were generated as described in *Materials and Methods*. C, role of TM2/TM7 interface in guanine nucleotide-sensitive high-affinity binding agonist to the α_{2A}AR. Membranes from transiently transfected HEK 293 cells expressing cDNAs encoding the α_{2A}AR structures shown were harvested and binding assays were performed as described in *Materials and Methods*. Each Gpp(NH)p curve represents the mean ± SE of three independent experiments performed in duplicate. The data are shown as the percentage of detectable specific [^{125}I]PIC agonist binding remaining after addition of various concentrations of Gpp(NH)p; 100% binding is defined as the specific binding of [^{125}I]PIC present in the absence of Gpp(NH)p. Nonspecific binding for [^{125}I]PIC is defined as that binding detected in the presence of 10 μM phentolamine, an α_{2A}AR antagonist. D to G, role of TM2/TM7 interface in Na^+-dependent allosteric modulation of ligand binding to the α_{2A}AR. Competition of epinephrine for [^{3}H]RX821002 antagonist binding was evaluated in the absence (solid lines and closed symbols) or presence (dashed lines and open symbols) of 100 mM Na^+ for wild-type (●), D79N (■), N422D (▲), and D79N/N422D (♦) α_{2A}AR structures in membrane preparations from transiently transfected HEK 293 cells. Incubations without Na^+ were maintained at constant tonicity using *N*-methyl D-glucamine$^+$ as the substitute for Na^+. Individual curves were fit with the use of Prism software (GraphPAD Software, San Diego, CA). To evaluate allosteric modulation of ligand binding by Na^+, an EC_{50} ratio was determined comparing the EC_{50} of epinephrine-mediated competition for [^{3}H]2821002 in the presence of Na^+ to that in the absence of Na^+. These results and subsequent statistical analyses are reported in Table 1.

μl SDS-sample buffer consisting of 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromphenol blue (with the addition of 2 mM Na-orthovanadate). This lysate was passaged twice through a 25-gauge needle and then placed on ice. Samples were then sonicated for 30 s and heated at 95°C for 5 min followed by centrifugation at 13,000*g* for 5 min. An aliquot (40 μl) was then subjected to 10% SDS-PAGE followed by Western analysis for using both anti-active and anti-total MAP kinase antibodies as described previously (Schramm and Limbird, 1999).

Assessment of Ligand-Dependent Receptor Density Up-Regulation. To assess the effect of agonist or antagonist occupancy on steady-state α_{2A}AR density, EcR-CHO cells transiently express-

ing WT or mutant α_{2A}AR were incubated in serum-free medium containing 0.1 mM ascorbate alone or ascorbate with 100 μM epinephrine or 10 μM idazoxan for 16 to 24 h. Cells were then washed three times with phosphate-buffered saline, pH 7.4, prewarmed to 37°C to facilitate removal of ligand. Triplicate wells of a six-well plate were then scraped and pooled in 1.2 ml of ice-cold buffer consisting of 25 mM glycylglycine, 40 mM HEPES, 5 mM EDTA, 5 mM EGTA, pH 8.0, 10 U/ml aprotinin, and 0.1 mM PMSF and homogenized with five up/down strokes of a 25-gauge needle on ice. Total cell lysate was then subjected to binding analysis using a saturating concentration of [^{3}H]RX821002 (10 nM). Nonspecific binding was defined as that binding detected in the presence of 10 μM phentolamine. Samples were normalized for milligrams of protein using a protein assay kit (Bio-Rad, Hercules, CA) as outlined above.

Assessment of Cell Surface Receptor Turnover. To assess cell surface receptor turnover, receptors were first "up-regulated" by overnight treatment with idazoxan to increase the sensitivity of the assay for the mutant receptors (Fig. 3C). After overnight treatment, cells were washed three times with 37°C PBS to wash out the idazoxan, twice on ice with 4°C PBS, and biotinylated at 4°C with 1 mg/ml sulfosuccinimidobiotin (sulfo-NHS-biotin; Pierce Chemical, Rockford, IL) in PBS. Cells were then transferred to 37°C serum free medium with or without receptor ligand (10 μM idazoxan or 100 μM epinephrine in the presence of 100 μM ascorbate) and placed in a 37°C, 5% CO_2 incubator. At the time points indicated in Fig. 3A, medium was aspirated from the cells and duplicate wells of a six-well plate were placed on ice and scraped into 500 μl/well of 4 mg/ml dodecyl-β-D-maltoside, 0.8 mg/ml cholesteryl hemisuccinate, 20% glycerol, 25 mM glycylglycine, 20 mM HEPES, 100 mM NaCl, 5 mM EGTA, pH 8.0, 0.1 mM PMSF, and 10 U/ml aprotinin (called DβM/CHS extraction buffer), pooled and passaged five times on ice through a 25-gauge needle. Cellular debris was cleared from solubilized protein via centrifugation at 13,000 rpm in a Microfuge at 4°C for 30 min. This supernatant, referred to as the detergent-solubilized preparation, was then incubated with 50 μl of streptavidin-agarose overnight at 4°C on an inversion wheel. The streptavidin-agarose was washed twice with 0.5 mg/ml dodecyl-β-D-maltoside, 0.1 mg/ml cholesteryl hemisuccinate in 25 mM glyclyglycine, 20 mM HEPES, 100 mM NaCl, 5 mM EDTA, pH 8.0 (called DβM/CHS wash buffer), and then eluted into 150 μl of SDS sample buffer with 15 min at 90°C. These samples were then subjected to SDS-PAGE and transferred to nitrocellulose in a buffer containing 10 mM CAPS, pH 11.0, and 10% methanol for 1.2 h at 1 A. Nitrocellulose was blocked in Tris-buffered saline with 1% Tween 20 containing 5% nonfat dry milk for 1 h at room temperature. HA-tagged receptor was then detected by blotting with a 1:1000 dilution of HA.11 primary antibody (Covance Research Products, Berkeley, CA) in blocking buffer followed by anti-mouse horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Piscataway, NJ). For semiquantification of Western analyses, films were digitized by scanning into Adobe Photoshop (Adobe Systems, Mountain View CA) and analyzed with NIH image software.

Assessment of Stability of Receptor Binding and Receptor Protein in Detergent-Solubilized Preparations. Transiently transfected COS M6 cells expressing wild-type or mutant α_{2A}AR were rinsed with PBS 48 h after transfection. Cells were then biotinylated at room temperature with 1 mg/ml sulfosuccinimidobiotin in PBS, as above. The biotinylation solution was then aspirated and cells were scraped on ice into ice-cold 15 mM HEPES, 5 mM EDTA, and 5 mM EGTA, pH 7.6 (with the addition of 10 U/ml aprotinin, 0.1 mM PMSF, 1 mg/ml soybean trypsin inhibitor, and 1 mg/ml leupeptin) and passaged five times up/down through a 25-gauge needle on ice. Lysates were then centrifuged at 40,000*g* for 15 min at 4°C. Pellets were resuspended on ice into DβM/CHS extraction buffer (with the addition of 10 U/ml aprotinin, 0.1 mM PMSF, 1 mg/ml soybean trypsin inhibitor and 1 mg/ml leupeptin). Receptor was solubilized by 10 up/down strokes in a Teflon/glass homogenizer on ice. Cellular debris was cleared from solubilized protein by centrifugation at 13,000 rpm in a Microfuge for 30 min at 4°C. The supernatant fraction was defined as the detergent-solubilized receptor. To assess receptor stability over time, enough detergent solubilized protein was added to the binding reactions to achieve 0.25–0.5 pmol of bound receptor at time 0 (immediately after solubilization and clearance from cellular debris). Change in functional binding capacity was followed as a function of time by keeping the detergent-solubilized receptors at 25°C and assaying the same volume of solubilized preparation per binding reaction at different time points (Wilson and Limbird, 2000). Specifically, the detergent-solubilized preparation was then warmed to 25°C. At the given time points, after incubation at 25°C, aliquots were removed and incubated with 40 μl streptavidin-agarose and 7.5 nM [^{3}H]yohimbine in DβM/CHS wash buffer (total reaction volume, 500 μl) at 4°C on an inversion wheel for 1 to 1.5 h. Beads were then washed twice with DβM/CHS wash buffer. The remaining beads were then directly added to scintillation cocktail (Aquasol) and counted on a Packard scintillation counter. The stability of receptor protein in these same samples was confirmed by Western analysis of the HA epitope in the α_{2A}AR proteins using the HA.11 antibody, as described above.

Results

To assess retrograde communication (G→R) between G proteins and WT and mutant α_{2A}-AR structures, we examined the ability of Gpp(NH)p to decrease receptor affinity for agonists, measured by the loss of trapability of the [^{125}I]PIC radiolabeled agonist as a function of increasing concentrations of the hydrolysis-resistant GTP analog, Gpp(NH)p (Ceresa and Limbird, 1994). As Gpp(NH)p concentrations are increased, the fraction of receptors in the higher affinity R-G complex ($K_{app} \cong 0.4$ nM) is diminished, as is the fraction of receptors that can be detected using the [^{125}I]PIC agonist radioligand (Baron and Siegel, 1990). The lower affinity interactions [R + G-Gpp(NH)p] with the receptor are not trapable using [^{125}I]PIC as the radioligand (Baron and Siegel, 1990; Ceresa and Limbird, 1994; Keefer et al., 1994) (Fig. 1C, schematic). As shown in Fig. 1C, more than 80% of the WT [^{125}I]PIC-specific binding detected is eliminated when incubations contain 100 μM Gpp(NH)p, consistent with the interpretation that the [^{125}I]PIC binding assay predominantly detects radioligand binding to high-affinity R-G complexes. Similar findings and interpretations have been obtained for radiolabeled agonist-binding to α_2-AR using [^{3}H]UK 14304 (Gerhardt et al., 1990) or to β_2-AR using other radiolabeled agonists (Stadel et al., 1980).

[^{125}I]PIC binding to the D79Nα_{2A}AR is relatively insensitive to the addition of Gpp(NH)p (Fig. 1C) (Ceresa and Limbird, 1994). In contrast, both the N422D and the D79N/N422D α_{2A}AR possess [^{125}I]PIC binding that is readily diminished in detectability in the presence of Gpp(NH)p (Fig. 1C). Compared with the WT α_{2A}-AR, the findings indicate that D79 is critical in regulating α_{2A}AR functional coupling to G proteins.

The *position* of the Gpp(NH)p curve reflects *not only* the affinity of Gpp(NH)p for the G proteins involved, *but also* the efficiency of G protein coupling to receptors, because Gpp(NH)p binding to the G protein (and consequent G protein conformational changes) leads to dissociation of the R-G complex, decreased receptor affinity for agonist, and thus diminished trapability of the [^{125}I]PIC binding (Fig. 1C, schematic). Because the [^{125}I]PIC binding detected for WT and

α_{2A}-AR mutant structures in these studies was pertussis toxin-sensitive (data not shown), the implication is that similar G proteins are involved in these interactions in the transient expression studies performed. Consequently, differences in the EC_{50} for Gpp(NH)p reflect differences in R-G coupling efficiency and retrograde G→R signaling from a similar pool of G proteins to different (WT versus mutant) receptor structures. Gpp(NH)p is more potent in decreasing high affinity agonist binding to the N422D [EC_{50} 64 ± 7.5 nM (mean ± SE); $n = 3$] and D79N/N422D α_{2A}AR (EC_{50} 22 ± 4 nM; $n = 3$) structures when compared with wild-type α_{2A}AR (EC_{50} 257 ± 39 nM (mean ± SE; $n = 3$; Fig. 1C). A leftward shift in the Gpp(NH)p concentration response curve for decreasing [^{125}I]PIC trapability denotes an *increased* efficiency with which Gpp(NH)p dislodges high efficiency R-G interactions and is consistent with the interpretation that the functional interface between the D79N/N422D α_{2A}AR or the N422D α_{2A}AR may be more fragile than between the WT α_{2A}AR and thus more easily disrupted by guanine nucleotides.

Allosteric Modulation of Ligand Binding by Na^+ Correlates with Membrane-Embedded Asp, but Not Asn, Residues. Agonist (epinephrine) competition for binding of the radiolabeled antagonist ([^{3}H]RX821002), in the presence or absence of Na^+, was used to evaluate allosteric modulation of ligand binding to the α_{2A}AR by monovalent cations (Tsai and Lefkowitz, 1978; Michel et al., 1980; Nunnari et al., 1987). As shown in Fig. 1D, the wild-type α_{2A}AR exhibits allosteric modulation, as manifested by a rightward shift of the epinephrine competition curve in the presence of Na^+ because of Na^+-evoked decreases in receptor affinity for agonists. This shift in the competition curve in the presence of Na^+ solely reflects a decrease in the receptor affinity for epinephrine, because [^{3}H]RX821002 seems to be insensitive to allosteric modulation in its affinity for α_{2A}AR (MacMillan et al., 1996) in contrast to Na^+-evoked increases in receptor affinity for the antagonist [^{3}H]yohimbine (Nunnari et al., 1987). Allosteric modulation of agonist binding by Na^+ is lost in the D79N α_{2A}AR, indicated by a lack of effect of Na^+ on epinephrine competition for [^{3}H]RX821002 (Fig. 1E), corroborating the role of aspartate D79 in allosteric modulation of ligand binding by cations in the α_{2A}AR (Horstman et al., 1990) and in a variety of other GPCR (Neve et al., 1991; Kong et al., 1993; Tian and Deth, 1993). Interchange of the aspartate in TM7 with the asparagine in TM7, creating the D79N/N422D α_{2A}AR double mutant, creates an intermediate phenotype of allosteric modulation of ligand binding (Fig. 1G). Not surprisingly, the single mutant N422D α_{2A}AR (Fig. 1F) also exhibits allosteric modulation of agonist binding by Na^+, because this structure contains an aspartate in both TM2 and TM7 that both could serve as negative counterions for the Na^+ cation. All of the α_{2A}AR mutants studied (i.e., D79N, N422D, or D79N/N422D) seem to possess a higher affinity for agonist than the wild-type α_{2A}AR, manifested as a shift to the left in the agonist competition curve in the absence of Na^+ [receptor structure EC_{50} ($n = 3$): WT, 9.5 ± 2 μM; D79N, 1.4 ±1 μM*; N422D, 1.0 ± 0.5 μM*; D79N/N422D, 1.6 ± 0.8 μM* (*$p < 0.05$ compared with WT) (Fig. 1, D-G)], as reported previously for the D79N mutation in the α_{2A}AR (Horstman et al., 1990; Lakhlani et al., 1997).

Impact of the Presumed α_{2A}AR Asp79(TM2)/Asn422(TM7) Interface on MAP Kinase Activation. Previous studies have demonstrated a selective inability of the D79Nα_{2A}AR to activate G protein $\beta\gamma$-dependent pathways (Surprenant et al., 1992), such as the activation of receptor-operated K^+ currents (Clapham and Neer, 1993) compared with α subunit-involved pathways such as inhibition adenylyl cyclase or voltage-gated Ca^{2+} currents (Surprenant et al., 1992; Lakhlani et al., 1996). Because the D79N mutation is also known to result in ablation of allosteric modulation of ligand binding (Horstman et al., 1990), it is reasonable to postulate a functional link between allosteric modulation of receptor conformation and activation of $\beta\gamma$-dependent pathways, corresponding to the postulated link between Na^+ modulation of receptor affinity and receptor-$\beta\gamma$ subunit interactions (Costa et al., 1992; Onaran et al., 1993).

GPCR-mediated activation of the MAP kinase cascade is thought to occur primarily through a $\beta\gamma$-dependent pathway, especially for the α_{2A}AR (van Biesen et al., 1995). As shown in Fig. 2, the wild-type α_{2A}AR is able to activate the MAP kinase in response to epinephrine in a concentration-dependent manner in HEK 293 cells. Activation of the MAP kinase is not detected for the D79Nα_{2A}AR, despite similar levels of D79N and mutant receptor expression as WT receptor in these experiments. Epinephrine activates MAP kinase via the N422Dα_{2A}AR, although with a trend of a reduced potency (note trend of rightward shift in the EC_{50} value for epinephrine to activate MAP kinase in N422D α_{2A}-AR-expressing cells compared with cells expressing the WTα_{2A}-AR) contrasting with the *increased* affinity for agonist ligand for this

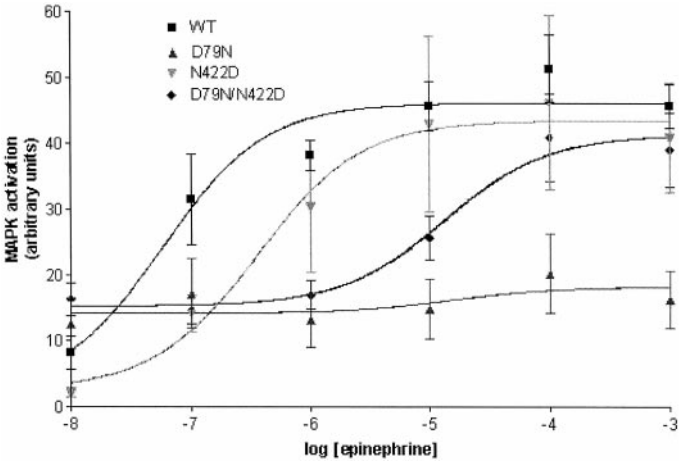

Fig. 2. The ability of various α_{2A}AR structures mutated at the presumed TM2/TM7 interface to activate MAP kinase. Transiently transfected HEK 293 cells expressing the α_{2A}AR structures shown were assayed 2 days after transfection for activation of MAP kinase. Activation was evaluated using incubation with epinephrine for 2 min as described in *Materials and Methods*. No stimulation by epinephrine was detected in nontransfected HEK 293 cells. Lysates were subjected to 10% SDS-PAGE followed by Western analysis using an antibody against the dually phosphorylated MAP kinase ("active MAP kinase"). Samples were then normalized for total MAP kinase in the same sample via Western analysis using an antibody that detects MAP kinase independent of its phosphorylation state. Westerns were developed using enhanced chemiluminescence. After Western analysis, the relative signal of the active (dually phosphorylated) and total MAP kinase was quantified using NIH Image Software. The data shown are the mean ± SE of four experiments. Epinephrine-mediated fold stimulation of MAP kinase for each receptor structure studied was determined by comparing the extent of MAP kinase activation in the presence of 1 mM epinephrine with that in the presence of no epinephrine. Results and subsequent statistical analysis as well as the EC_{50} of MAP kinase activation for each receptor studied are reported in Tables 1 and 2.

receptor structure (Fig. 1, D and G). Taken together, these observations indicate that the efficacy of N422Dα_{2A}AR coupling to G proteins is less than that of the wild-type α_{2A}AR, corroborating the interpretations of the increased sensitivity of ^{125}I-PIC binding to Gpp(NH)p for the N422Dα_{2A}AR (Fig. 1C). Interchange of residues 79 and 422, creating the D79N/N422D double mutant α_{2A}AR, also permits MAP kinase activation by epinephrine in a dose-dependent manner, but with a diminished potency compared with the wild-type α_{2A}AR (Fig. 2 and Tables 1 and 2). This finding is again consistent with the interpretation of findings in Fig. 1 that the D79N and N422D residues contribute to an interface between TM2 and TM7 that regulates receptor signaling properties.

Mutation of Residues 79 or 422 Alters Cell Surface Receptor Turnover and Results in Ligand Modulation of Receptor Turnover and Density. Recently, we demonstrated that the D79N α_{2A}AR manifests a higher rate of cell-surface turnover and that this turnover can be slowed by receptor structural stabilization with either agonist or antagonist occupancy, resulting in steady-state receptor density up-regulation (Wilson and Limbird, 2000). The N422Dα_{2A}AR, despite retention of allosteric modulation of ligand binding by Na^+ (Fig. 2I), possesses a surface $t_{1/2}$ of <3 h compared with the 13 ± 1.0 h surface $t_{1/2}$ of wild-type α_{2A}AR (Fig. 3A). The N422Dα_{2A}AR also exhibits receptor density up-regulation by antagonist but not by agonist (Fig. 3C); similarly, antagonist, but not agonist, occupancy of the receptor slows cell surface receptor turnover (Fig. 3B).

Interchange of residues 79 and 422 (D79N/N422D) of the α_{2A}AR slows the surface $t_{1/2}$ to 4.7 ± 0.3 h for the D79N/N422D α_{2A}AR (Fig. 3A) compared with the <3 h $t_{1/2}$ characteristic of the single mutant N422D α_{2A}AR. Turnover of the D79N/N422Dα_{2A}AR is slowed by occupancy of the mutant α_{2A}AR with either agonist or antagonist (Fig. 3B) and both agonist and antagonist similarly up-regulate receptor density of the D79N/N422Dα_{2A}AR (Fig. 3C), recapitulating findings for the D79Nα_{2A}AR (Fig. 3, B and C). Thus, although the D79N/N422D double mutant exhibits allosteric modulation by Na^+ and functional α_{2A}AR-G protein coupling (Fig. 1), receptor stabilization on the cell surface is not fully restored to that characteristic of the WT α_{2A}AR, analogous to findings for MAP kinase activation by epinephrine at the D79N/N422D α_{2A}AR.

Consequences of Mutations At the Presumed Asp79(TM2)/Asn422(TM7) Interface on α_{2a}AR Conformational Stability.

Accelerated cell surface receptor turnover, in the context of ligand-mediated increases in receptor density expression in cells, can be a manifestation of the structural or conformational lability of a receptor (Wilson and Limbird, 2000). A direct measure of conformational lability in mutant GPCRs is a comparison of the rate of loss of receptor binding capability (a reflection of conformational/structural stability) with the rate of loss of receptor protein (a reflection of protein stability) in detergent-solubilized preparations (Gether et al., 1997; Wilson and Limbird, 2000). As shown in Fig. 4, the N422D α_{2A}AR is extremely structurally/conformationally unstable compared with wild-type or even D79N α_{2A}AR (Fig. 4), which might be expected given the presumed juxtaposition of two negative charges within the bilayer of the α_{2A}AR structure. Placing the D79N mutation in the N422D structure (D79N/N422D) creates an α_{2A}AR structure with an intermediate phenotype for conformational stability compared with either single mutation (D79N or N422D) alone (Fig. 4), based on the approximate $t_{1/2}$ of functional binding capacity in detergent solution of 10 min for N422D, 1.5 h for D79N and 20 min for D79N/N422D.

Additional Mutagenesis at Residue 79 Reveals the Critical Importance of the Aspartate 79 Residue Per Se in Regulating Receptor Cell-Surface Residence Time and Structural Stability. The observation that interchanging the aspartate in TM2 with the asparagine in TM7, generating the D79N/N422D α_{2A}AR double mutant, diminishes cell-surface residence time and enhances structural lability in detergent solution when compare to the wild-type α_{2A}AR implies that simply complementing the asparagine at 79 with a negative charge at this TM2/TM7 interface (N422D) is not sufficient to impart functional properties identical with that of the wild-type receptor. To explore this interpretation further, two additional mutant α_{2A}AR structures were examined. The D79E α_{2A}AR substitutes the negatively charged

TABLE 1

A comparison of the functional and structural properties of WT and mutant α_{2a}AR structures: G protein coupling and allosteric modulation by Na^+

For statistical analysis, analysis of variance was used followed by a Student-Newman-Keuls multiple comparisons post test comparing each receptor with that of WT. The EC_{50} value for Gpp(NH)p for each receptor structure was determined using a competition model of nonlinear regression to fit each individual experiment. The EC_{50} values reported represent the mean ± SE of three independent experiments. The EC_{50} ratio was determined using nonlinear regression to fit individual curves in the presence or absence of Na^+ followed by creating a ratio EC_{50} (+ Na^+)/EC_{50}(− Na^+). The EC_{50} ratio reported represents the mean ± SE of three independent experiments. The epinephrine-elicited fold stimulation of MAP kinase was determined for each receptor structure studied by dividing the MAP kinase activation at 1 mM epinephrine by the MAP kinase activation in the presence of no epinephrine. The fold stimulation expressed represents the mean ± SE of four independent experiments. The EC_{50} of MAP kinase stimulation was determined for each receptor studied using sigmoidal dose-response non-linear regression fit (using GraphPAD Prism software) of each individual experiment (dose-response curve). The EC_{50} values expressed represent the mean ± SE of four independent experiments.

Receptor Structure	Inhibition of [^{125}I]PIC Binding by 0.1 mM Gpp(NH)p	EC_{50} for Gpp(NH)p	Agonist Competition for ^{3}H Antagonist: EC_{50} Ratio	Epinephrine-Activated MAP Kinase Stimulation	EC_{50} for Epinephrine Activation of MAP Kinase
	%	*μM*		*fold*	*μM*
WT	80 ± 2	0.26 ± 0.04	2.4 ± 0.23	5.1 ± 0.6	0.14 ± 0.06
D79N	15 ± 3	N.D.	1.02 ± 0.03***	1.05 ± 0.05***	N.D.
N422D	80 ± 2	0.064 ± 0.008**	2.8 ± 0.21	5.5 ± 0.8	87 ± 40
D79N/N422D	70 ± 3	0.022 ± 0.004***	1.8 ± 0.03*	2.7 ± 0.12**	18 ± 10
D79Q	10 ± 5[a]	N.D.[a]	N.D.	N.D.	N.D.
D79E	90 ± 2[a]	0.07 ± 0.009[a]***	N.D.	N.D.	N.D.

N.D., not determined.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
[a] Ceresa and Limbird (1994).

aspartate with a negatively charged glutamate at this TM2/TM7 interface. Previous studies from our laboratory have demonstrated that the D79E α_{2A}AR couples to G proteins and is allosterically modulated by cations in a manner indistinguishable from the WT α_{2A}AR (Ceresa and Limbird, 1994). However, mutation of aspartate to glutamate does more than simply substitute a negative charge with another, but also extends the presumed negative charge by one methylene group. Another mutation of residue 79, the D79Q α_{2A}AR, previously demonstrated to diminish G protein coupling efficiency and eliminate allosteric modulation by cations (Ceresa and Limbird, 1994), also was examined. The rate of cell-surface turnover of both the D79E and D79Q α_{2A}AR is more rapid than that of the wild-type α_{2A}AR (Fig. 3A). Additionally, both the D79E and D79Q α_{2A}AR are up-regulated in receptor density after incubation with either agonist or antagonist in intact cells (Fig. 3C), presumably because of ligand-mediated receptor stabilization and slowing of cell-surface receptor turnover (Fig. 3B).

Analysis of the structural stability of the D79E and D79Q α_{2A}ARs reveals that even the presumed conservative substitution of aspartate 79 with glutamate results in accelerated loss of binding activity in detergent compared with wild-type α_{2A}AR (Fig. 4). Interestingly, the rate of loss of binding capability in detergent for the D79E α_{2A}AR parallels the rate of loss for the D79N/N422D α_{2A}AR. These are important findings, because they indicate that allosteric modulation by cations and coupling to G proteins, both of which are true for the D79E and D79N/N422D α_{2A}ARs, do not necessarily rely on or predict a conformationally/structurally stable receptor structure [(Ceresa and Limbird, 1994) and Figs. 1–3].

Discussion

Cell-surface receptor expression is necessary for surface-mediated GPCR signaling. Conformational stability contributes to maintaining cell surface receptor expression of GPCR in general and the α_{2A}AR in particular. Mutant α_2-AR receptors lacking conformational stability exhibit a faster removal from the cell surface that can be slowed by receptor occupancy with ligand [Fig. 3 and 4 and (Wilson and Limbird, 2000)]. Previous investigations have focused on ligand mediated up-regulation of mutant GPCRs through either increased cell-surface delivery (Morello et al., 2000) or increased cell-surface residence time (Wilson and Limbird, 2000). The present study suggests that the conserved D79(TM2)/N422(TM7) interface contributes to conformational stability of the α_{2A}AR, and, because of the conserved nature of this interface, such findings may be generalizable to other GPCRs. The multiple functional and structural properties evaluated in this study for mutations at this interface are summarized in Tables 1 and 2.

Considerable modeling of the predicted interactions among amino acid side chains in the TM spans of GPCR has been undertaken, particularly for receptors that bind monoamines (Sealfon et al., 1995; Mizobe et al., 1996; Gether and Kobilka, 1998). These models, developed to describe a molecular basis for experimental data (Zhou et al., 1994; Sealfon et al., 1995), suggest that TM2 and TM7 are in near each other and that a hydrogen bonding network, including an aspartate in TM2 (D79 of the α_{2A}AR) and an asparagine in TM 7 (N422 of the α_{2A}AR), regulates receptor activation (Fig. 1, A and B). Therefore, to interpret our results in the context of the α_{2A}-AR, it was first important to evaluate mutations at this interface with regard to receptor activation.

Allosteric modulation of ligand binding to the α_{2A}AR seems to be dependent on a negative charge at the TM2/TM7 interface. All receptor structures that possess a negative charge in either TM2 (wild-type), TM7 (D79N/N422D), or both TM2 and TM7 (N422D) result in allosteric modulation by monovalent cations (Fig. 1, Tables 1 and 2). The ability of receptor to be allosterically modulated by cations correlates with G protein coupling efficiency, because receptors that undergo allosteric modulation (wild-type, N422D, or D79N/N422D) also exhibit high affinity guanine nucleotide-sensitive agonist binding (Fig. 1C and Table 1). In contrast, receptors that lack allosteric modulation by cations (D79N) do not possess guanine-nucleotide sensitive agonist binding (Fig. 1, C and D; Table 1), a measure of retrograde coupling of G proteins to cognate receptors (Ceresa and Limbird, 1994). The ability of receptors to couple to G proteins in membrane preparations correlates with the ability of receptor to activate MAP kinase in intact cells, a measure of anterograde receptor to G protein coupling. Thus, receptors that undergo modulation of ligand binding by cations (wild-type, N422D, and D79N/N422D α_{2A}ARs) also activate the MAP kinase cascade; those that lack allosteric modulation by cations or regulation of agonist binding by Gpp(NH)p (e.g., the D79N α_{2A}AR in Fig. 1, C and

TABLE 2

Comparison of the functional and structural properties of WT and mutant α_{2A}AR structures: receptor cell-surface residence time and conformational stability

For statistical analysis, analysis of variance was used followed by a Student Newman-Keuls multiple comparisons post test comparing each receptor with that of WT.

Receptor Structure	Surface $t_{1/2}$	Ligand-Dependent Surface Stabilization	Ligand-Dependent Up-Regulation	Loss of Binding Capacity after Detergent Solubilization
	h			
WT	13 ± 1.0	Little or none	Little or none	Stable
D79N	5.7 ± 0.9*	Increased by ag/antag	Increased by ag/antag	Rapid loss ($t_{1/2}$ ~ 2 h)
N422D	<3*	Increased by antag only	Increased by antag only	Very rapid loss ($t_{1/2}$ < 15 min)
D79N/N422D	4.7 ± 0.3*	Increased by ag/antag	Increased by ag/antag	More rapid loss ($t_{1/2}$ ~ 15 min)
D79Q	3.7 ± 0.2*	Increased by ag/antag	Increased by ag/antag	Rapid loss ($t_{1/2}$ ~ 2 h)
D79E	3.1 ± 1.4*	Increased by ag/antag	Increased by ag/antag	More rapid loss ($t_{1/2}$ ~ 15 min)

* $p < 0.05$.

E) do not. Collectively, these results regarding allosteric modulation by Na^+ and receptor-G protein coupling correlate with data previously obtained for other GPCR with regards to modulating the structure of GPCR at the presumed TM2/TM7 interface (Table 1).

The conserved TM2/TM7 interface also seems to play some role in regulating receptor cell surface residence time. Thus, mutation of D79 to N, E, or Q in the α_{2A}AR is paralleled by faster cell surface receptor turnover (Fig. 3) and structural instability (Fig. 4), despite differing consequences on receptor activation (Wilson and Limbird, 2000). Mutation of TM7 at the TM2/TM7 interface to create a receptor with presumably apposing negative charges (i.e., the N422D α_{2A}AR) leads to an extremely unstable α_{2A}AR molecule, as measured by multiple independent lines of evidence including: 1) accelerated rate of loss functional binding capacity after detergent solubilization (Fig. 4); 2) increased cell surface receptor turnover (Fig. 3A); 3) ligand-stabilized attenuation of receptor turnover (Fig. 3B); and 4) ligand-mediated, steady-state up-regulation of receptor density (Fig. 3C). It is probable that the extreme structural instability of the N422Dα_{2A}AR results from apposition of two negatively charged residues near each other within the transmembrane domain core of the α_{2A}AR. It is interesting that only antagonist occupancy, but not agonist occupancy, serves to stabilize the surface residence of the N422Dα_{2A}AR, resulting in dramatic up-regulation of N422Dα_{2A}AR density. It is possible that antagonist occupancy of the receptor stabilizes a conformation in which the interaction of the negatively-charged residues D79(TM2) and N422D(TM7) does not occur. In contrast, the agonist epinephrine, by stabilizing a distinct conformation, may foster a more direct interaction of these two residues, resulting in a considerably more unstable receptor structure, thus accounting for the lack of effect of epinephrine on cell surface receptor stabilization and resultant up-regulation of steady state receptor density. Such an interpretation is consistent with other lines of evidence that agonists and antagonists serve to stabilize differing receptor conformations (Gether and Kobilka, 1998). Placing the D79N mutation within the N422Dα_{2A}AR structure restores some structural stability,

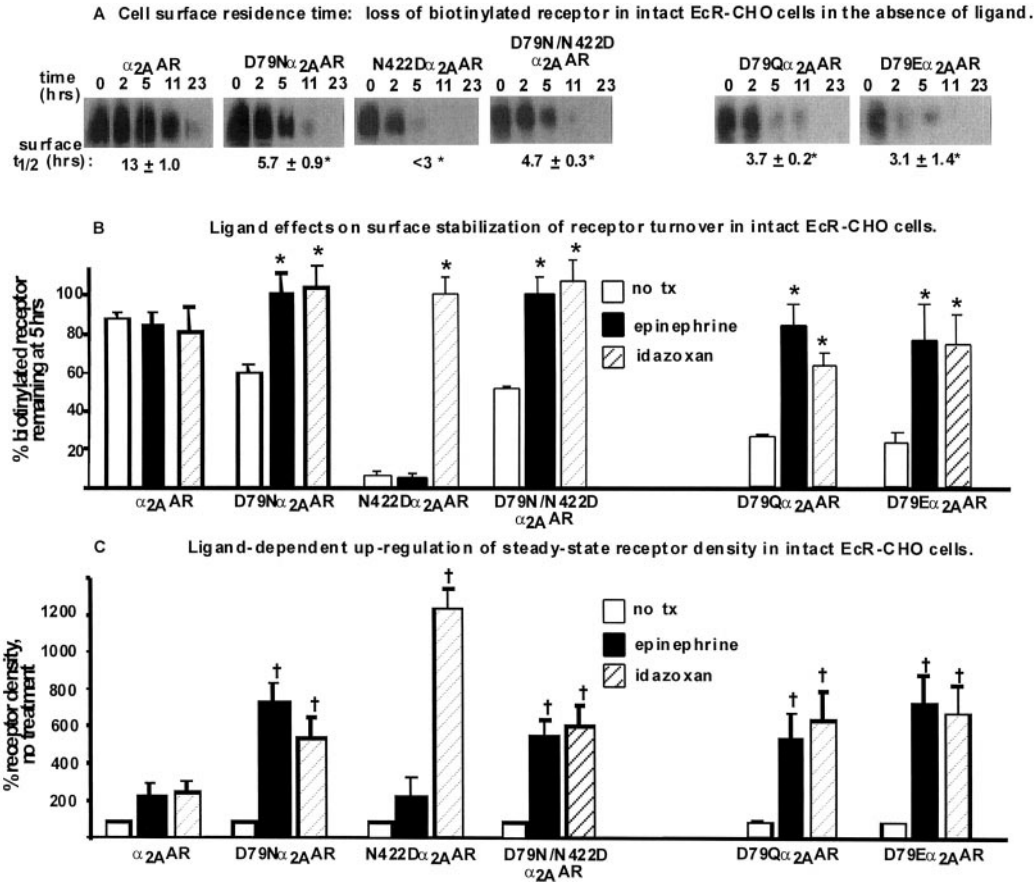

Fig. 3. Mutation of residues 79 and/or 422 of the α_{2A}AR results in enhanced cell surface turnover that can be slowed with ligand occupancy of the mutant receptors. A, transiently-transfected EcR-CHO cells expressing cDNAs encoding the α_{2A}AR structures shown were evaluated for surface turnover of expressed receptor using a cell surface biotinylation strategy (see under *Materials and Methods*). ANOVA reveals a $p < 0.05$ and * indicates a $p < 0.05$ using a Student-Newman-Keuls multiple comparisons post test comparing wild-type with 79N, N422D, D79E, D79Q, and D79N/N422D α_{2A}AR surface $t_{1/2}$ calculated using nonlinear regression curve fitting of semiquantified Western analysis (mean ± SE, $n = 3$). B, analysis of ligand effects on surface stabilization of wild-type and mutant receptors (mean ± SE, $n = 4–6$). ANOVA reveals a $p < 0.05$ and * indicates a $p < 0.05$ using a Student-Newman-Keuls post test comparing no treatment with treatment with ligand for a given receptor. C, transiently transfected EcR-CHO cells were incubated with agonist (100 μM epinephrine) or antagonist (10 μM idazoxan) overnight and assayed for receptor expression. Receptor expression was evaluated with [^{3}H]RX821002 binding analysis. Shown is the average of three independent experiments for binding (mean ± SE; $n = 3$). ANOVA reveals a $p < 0.05$ and † indicates a $p < 0.05$ using a Student-Newman-Keuls multiple comparisons post test comparing wild-type with D79N, N422D, and D79N/N422D α_{2A}AR density after overnight treatment. The receptor density in the absence of overnight treatment (defined as 100%) was as follows (mean ± SE in pmol binding/mg of protein): wild-type, 1.4 ± 0.2; D79N, 0.42 ± 0.2; N422D, 0.2 ± 0.05; D79N/N422D, 0.25 ± 0.05; D79E, 0.4 ± 0.1; D79Q, 0.5 ± 0.2.

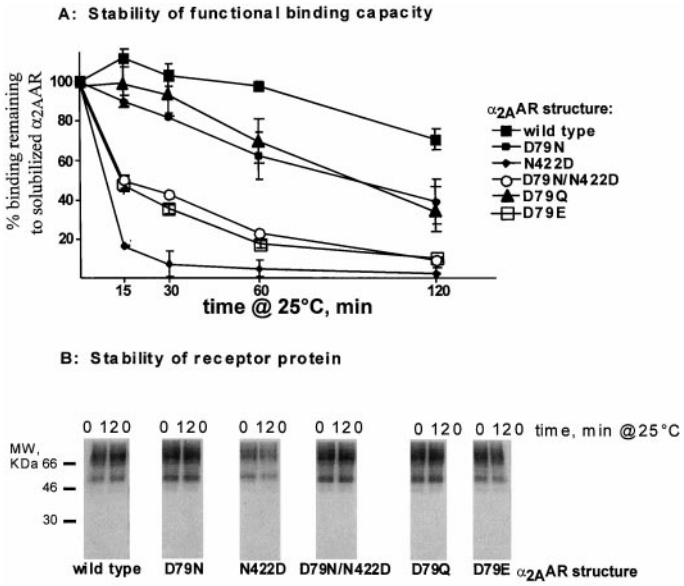

Fig. 4. Impact of mutations at the presumed TM2/TM7 interface on conformational stability of the α_{2A}AR. A, conformational stability was measured by monitoring functional receptor binding capacity as a function of time at 25°C. Receptors were extracted from COS M6 cells as described under *Experimental Procedures* and binding to detergent-solubilized preparations was monitored using [^{3}H]yohimbine as the radioligand. Binding corresponded to 0.25 to 0.5 pmol of receptor at time 0. Shown is the mean percentage ± SE from three independent experiments. B, Western analysis reveals that no receptor degradation occurs for any of the structures evaluated during the 120-min postsolubilization incubation. These findings indicate that the loss of binding capacity seen in A is caused by structural instability of the receptor molecule and not degradation of the receptor protein. The wild-type, D79N, N422D, D79E, D79Q, and D79N/N422D α_{2A}AR run at 66 kDa. The lower molecular mass bands probably represent incompletely processed receptor in COS M6 cells transiently expressing these receptor structures.

which manifests as a decrease in the loss of functional binding capacity after detergent solubilization (Fig. 4) and a slower surface turnover in intact cells (Fig. 3A), presumably because of removal of one of the negative charges at this interface.

The properties of the D79E and D79N/N422D α_{2A}AR structures, compared with those of wild-type α_{2A}AR, reveal the critical importance of the precise structural localization of the negative charge on the side chain of residue 79 in regulating receptor structural stability. Although the D79E α_{2A}AR couples to G proteins and its binding of agonist and antagonist ligands is modulated by monovalent cations in a way that is indistinguishable from the WT α_{2A}AR (Ceresa and Limbird, 1994), the D79E α_{2A}AR exhibits structural instability like that of the D79N/N422D α_{2A}AR double mutant (Fig. 4). Thus, although a negative charge at residue 79 is necessary to permit functional activity of the α_{2A}AR, it is not sufficient to afford all of the properties of the wild-type receptor, including intrinsic conformational/structural stability and prolonged receptor cell-surface residence time (Table 2).

Our data are consistent with a proximity of the TM2 and TM7 transmembrane helices, by analogy with the amine binding $5HT_{2A}$ receptor and the peptide binding GnRH-R and μ-opioid receptor (Zhou et al., 1994; Sealfon et al., 1995; Flanagan et al., 1999; Xu et al., 1999). It is probable that this interface involves multiple independent or interdependent contact sites. However, our data are not necessarily consistent with a direct charge pairing of D79 in TM2 with N442 in TM7, because "swapping" of these residues via mutagenesis does not create a receptor structure (D79N/N422D) with functional or stability properties indistinguishable from the wild-type receptor. This interpretation is consistent with recent crystallographic findings for another GPCR, rhodopsin, suggesting that a molecule of water bridges these two residues (Palczewski et al., 2000). Nonetheless, our data are consistent with the interpretation that the two residues do contribute to this TM2-TM7 interface, which regulates multiple biochemical properties of the α_{2A}-AR (Tables 1 and 2).

For the α_{2A}AR, possessing a negative charge in TM2(D79), TM7 (D79N/N422D), or both TM2 and TM7 (N422D) is sufficient to impart allosteric modulation of ligand binding by monovalent cations, coupling to G proteins, and activation of MAP kinase activity. However, this interface also is essential for maintaining intrinsic receptor conformational/structural stability. Receptor structural stability seems to be more sensitive than receptor activation to structural modification by mutation of residues at this interface, borne out by the observation of the extreme structural instability of the N422D α_{2A}AR as well as structural instability of the conservatively substituted D79E α_{2A}AR. Taken together, these data suggest that this TM2/TM7 interface in GPCRs is involved in two distinct phenomena: receptor activation (measured by G protein coupling and activation of downstream effectors) and receptor conformational stability (measured directly by following receptor binding in detergent solution over time and indirectly as receptor cell-surface residence time) (Tables 1 an 2). Elucidation of sites critical for regulating or maintaining GPCR conformational/structural stability as well as cell-surface expression is an important step in providing novel therapeutic/pharmacological modulation of GPCR. This highly conserved TM2/TM7 interface seems to be one such critical site, especially because of its role in regulating receptor activation.

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