Role for the Third Intracellular Loop in Cell Surface Stabilization of the α2A-Adrenergic Receptor*

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Stephen W. Edwards‡ and Lee E. Limbird$†

From the Department of Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37232

Previous studies have shown that α2A-adrenergic receptor (α2A-AR) retention at the basolateral surface of polarized MDCKII cells involves its third intracellular (3i loop). The present studies examining mutant α2A-ARs possessing short deletions of the 3i loop indicate that no single region can completely account for the accelerated surface turnover of the Δ3i α2A-AR, suggesting that the entire 3i loop is involved in basolateral retention. Both wild-type and Δ3i loop α2A-ARs are extracted from polarized Madin-Darby canine kidney (MDCK) cells with 0.2% Triton X-100 and with a similar concentration/response profile, suggesting that Triton X-100-resistant interactions of the α2A-AR with cytoskeletal proteins are not involved in receptor retention on the basolateral surface. The indistinguishable basolateral t1/2 for either the wild-type or nonsense 3i loop α2A-AR suggests that the stabilizing properties of the α2A-AR 3i loop are not uniquely dependent on a specific sequence of amino acids. The accelerated turnover of Δ3i α2A-AR cannot be attributed to alteration in agonist-elicited α2A-AR redistribution, because α2A-ARs are not down-regulated in response to agonist. Taken together, the present studies show that stabilization of the α2A-AR on the basolateral surface of MDCKII cells involves multiple mechanisms, with the third intracellular loop playing a central role in regulating these processes.

The α2A-adrenergic receptor (α2A-AR)1 is a member of a large family of G protein-coupled receptors that are predicted to have seven transmembrane-spanning regions (1, 2). Three subtypes of α2-ARs exist and couple to members of the Gq and G12 class of G-proteins to mediate a variety of physiological responses (3, 4).

Receptor localization and stabilization on the cell surface of target cells are two critical contributors to the sensitivity and extent of signaling by G protein-coupled receptors. There is a growing body of evidence that discrete localization of G protein-coupled receptors may play a role in specificity of signaling by these receptors (5–8). A precedent already exists for the micro-compartmentation of signaling molecules such as protein kinase C (9), cAMP-dependent protein kinase (10), Ca2+/calmodulin-dependent protein kinase II (11), kinases involved in the yeast mating response (12), and NO synthase (13, 14) by interaction of these effector molecules with signaling "scaffold" proteins.

In polarized cells, receptor localization is essential for vectorial information transfer, as occurs for α2-AR regulation of Na+ and H2O transport in renal (15) and intestinal (16, 17) epithelial cells. Madin-Darby canine kidney (MDCKII) cells cultured in Transwell culture dishes have provided an excellent model system for polarized renal epithelial cells. The localization of the α2A-AR subtype on the basolateral surface of these cells (18) recapitulates the basolateral localization of this receptor in vivo, based on physiological (19) and pharmacological (20) data.

Examination of molecular regions of the three α2A-AR subtypes in polarized MDCKII cells indicates that basolateral targeting of these receptors involves sequences in or near the membrane bilayer (18, 21, 22). In contrast, the large third intracellular loop of the receptor appears to play a role in stabilizing the α2A-AR on the plasma membrane, because mutant α2A-ARs that lack 119 amino acids from the large third intracellular loop (Δ3i loop α2A-AR) have a cell surface half-life (t1/2) of 4.5 h compared with a t1/2 of 10 h for the wild-type α2A-AR (21). The present studies have explored the structural features of the Δ3i loop responsible for stabilizing the α2A-AR on the plasma membrane of MDCKII cells.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (6000 Ci/mmol), [3H]yohimbine (70–90 Ci/mmol), [125I]iodoclonidine (2200 Ci/mmol), [3H]Express protein labeling mix (1200 Ci/mmol), [3H]methoxyinulin (Ci/mmol), and [3H]3DATP (1389Ci/mmol) were purchased from NEN Life Science Products. Phentolamine was kindly provided by CIBA Pharmaceutical Co.125I-Rau-AzPEC (17-hydroxy-20-yohimbane-16-(N-4-azido-3-[125I]iodophenyl)carboxamide) was synthesized in our laboratory by the method of Lanier et al. (23). Biotin hydrazide, Sulfo-NHS-biotin, and streptavidin-agarose were purchased from Pierce. The protein A-purified 12CA5 monoclonal antibody was purchased from the Berkeley Antibody Co. Epinephrine was obtained from Sigma, and ascorbic acid was purchased from Fisher.

Cell Culture—Madin-Darby canine kidney cells (MDCKII) were obtained from Enrique Rodriguez-Boulan (Cornell Univ. New York, NY) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Sigma), 100 units/ml penicillin, and 100 mg/ml streptomycin (referred to as complete Dulbecco’s modified Eagle’s medium) at 37 °C, 5% CO2. For polarity experiments, MDCK II cells were seeded at a density of 1 × 106 cells/24.5-mm polycarbonate membrane filter (Transwell chambers, 0.4-μm pore size, Costar, Cambridge, MA) and cultured for 5–8 days with a change of medium every 1–2 days. Before each experiment, the integrity of the monolayer was assessed by adding [3H]methoxyinulin to the apical medium and monitoring its leak after a 1-h incubation at 37 °C from the apical to the basolateral compartment by sampling and counting the basolateral medium in a scintillation counter (Packard Tri Carb). Chambers with greater than 5% leak/h were not evaluated.

Construction of Mutant α2A-AR Permanently Expressing Cell Lines—Generation and characterization of Δ3i loop α2A-AR (Δaa240–359) has...
been previously described (21, 24). Briefly, site-directed mutagenesis was used to create a novel NdeI restriction enzyme site in the region of the α2A-AR cDNA encoding the C-terminal end of the putative third intracellular (3i) loop. Cleavage of this mutant α2A-AR cDNA with NdeI restriction enzymes removes the DNA fragment encoding amino acids 240–359. This 3i loop mutant receptor has 36 amino acids linking transmembrane domains 5 and 6 of the α2A-AR as shown in Fig. 1A.

Oligo-directed mutagenesis in M13 phage was utilized to create incremental deletions of the predicted third intracellular loop of the α2A-AR. Single oligos were designed against sequences flanking those encoding the amino acids selected for each deletion. The deletion mutations were confirmed by deoxyxynucleotide sequencing and then subcloned into the pCMV4 mammalian expression vector. Deletions corresponding to DNA encoding the following amino acids were made in this manner: Δaa252–267, Δaa286–303, Δaa252–267, Δaa252–267, Δaa252–267, Δaa252–267, Δaa252–267, Δaa315–326, Δaa315–326, Δaa327–340, Δaa327–340, and Δaa252–267. Fig. 1 provides a schematic diagram of the regions encoded by these deleted amino acids.

The nonsense loop was designed by taking advantage of the method used for making the original Δaa240–359 (3i loop α2A-AR). Because two NdeI enzyme sites were used to remove the 3i loop, it was possible to subclone this segment of the gene back into the receptor in two orientations. The correct orientation produced a receptor that corre- sponding to N-terminal hemagglutinin epitope (YPYDVPDYA) to which antibodies are available commercially (Berkeley Antibody Co.). These plasmid constructs were verified by double-stranded DNA sequencing through the region of the mutation. COS-7 cells were transiently transfected with the plasmid DNA encoding the wild-type and mutant α2A-ARs, and membranes from the transient transfecants were assayed for [3H]yohimbine binding before developing permanent MDCK cell lines expressing these mutant α2A-ARs. MDCK cell lines permanently expressing the wild-type or mutant α2A-ARs were created as described previously (18) (Table I).

Determination of the Half-life of Wild-type or Mutant α2A-AR on the Basolateral Membrane—To determine α2A-AR half-life on the basolateral surface, a metabolic labeling strategy was employed. MDCK cells expressing wild-type or mutant α2A-ARs were incubated with [35S]Cys/Met (“pulse”) and then incubated for various periods of time (“chase”) before isolation of basolateral α2A-AR using sequential biotinylation, extraction, immunosolation, and streptavidin-agarose chromatogra-
to 1% Triton X-100, the residual cellular material, operationally defined as “Triton shells,” was scraped into 200 μl of RIPA buffer. A set of control Transwells were subjected to the same procedure, except that each successive buffer contained no Triton X-100. The final RIPA extraction buffer from each well was centrifuged at 13,000 g for 5 min. Subsequently, the supernatant was transferred to a 0.6-ml Eppendorf tube, and the wells were washed with 200 μl of RIPA buffer. All extracts were brought up to a final volume of 500 μl with RIPA buffer, and biotinylated proteins were isolated using streptavidin-agarose chromatography. After an overnight incubation, the streptavidin beads were washed with 1X streptavidin-agarose wash buffer. The biotinylated proteins were eluted with 1X streptavidin-agarose wash buffer. All extracts were loaded onto a 10% SDS–polyacrylamide gel. The dried gels were exposed to a preflushed Kodak film for 3–5 days. The receptor was identified as a radioactive band by autoradiography. After an overnight incubation, the streptavidin beads were washed with 1X streptavidin-agarose wash buffer. The biotinylated proteins were isolated using streptavidin-agarose chromatography. After an overnight incubation, the streptavidin beads were washed with 1X streptavidin-agarose wash buffer. The biotinylated proteins were eluted with 1X streptavidin-agarose wash buffer. All extracts were loaded onto a 10% SDS–polyacrylamide gel. The dried gels were exposed to a preflushed Kodak film for 3–5 days. The receptor was identified as a radioactive band by autoradiography.

RESULTS AND DISCUSSION

No Small Region in the α2A-AR Third Intracellular Loop Contains All of the Necessary Information for Stabilization of the Receptor on the Cell Surface—We observed previously that deletion of 119 amino acids from the 3i loop of the α2A-AR (amino acids 240–359) generates a structure (Δ3i α2A-AR) that has a basolateral t½ of ~4.5 h compared with 10–12 h for the wild-type α2A-AR in polarized MDCKII cells. By analogy with the ability of a 21-amino acid insert into the short (D2S) dopamine receptor to create the long dopamine receptor isofrom (D2L) and dramatically slow the rate of sequestration (27), the 3i loop of the α2A-AR was examined to determine whether a single small amino acid sequence could account for the stabilization of the receptor.

Five ~20 amino acids deletions were made within the α2A-AR 3i loop, as shown schematically in Fig. 1A. Demarcation of the regions selected for individual deletions was based on secondary structural predictions of Chou and Fasman analysis (52); for example, Δaa286–303 and Δaa315–326 are predicted by this analysis to form amphipathic α helices. In addition, the Δaa286–303 removes the LEESSSS sequence recognized for phosphorylation by G protein-coupled receptor kinases (28, 29). This was of interest because G protein-coupled receptor kinase-mediated phosphorylation of these receptors promotes association with arrestins that have been shown to act as adaptors and recruit some G protein-coupled receptors to clathrin-coated pits (30–32).

The surface stability for each of the α2A-AR structures examined (Fig. 1A) was determined by pulse/chase metabolic labeling strategies and isolation of α2A-AR on the basolateral surface by sequential biotinylation and streptavidin-agarose isolation of detergent-solubilized receptor (see “Experimental Procedures”). As shown in Fig. 1B, Δaa286–303 α2A-AR does not mimic the accelerated turnover characteristic of the Δ3i loop α2A-AR. Similar studies were performed for the other deletions shown in Fig. 1A and are summarized in Fig. 1C. Although three of the deletions may affect α2A-AR residence time on the cell surface somewhat, none of the smaller deletions within the 3i loop appear to mimic the accelerated turnover characteristic of the Δ3i loop α2A-AR. These data suggest that multiple noncontiguous sequences or bulk size of the 3i loop determine the residence time of α2A-AR on the cell surface.

Direct Cytoskeletal Interactions Do Not Appear to Account for Stabilization of the α2A-AR via Its 3i Loop—One mechanism that might account for stabilization of the α2A-AR on the basolateral surface is interaction with the cytoskeleton through the 3i loop or the cytoskeletal cytoskeleton through the 3i loop. In this case, accelerated turnover of the Δ3i loop α2A-AR could result from loss of these direct cytoskeletal interactions. We utilized differential sensitivity to extraction by Triton X-100 as an indicator of direct and stable association with the cytoskeleton (33–35). This approach has been informative in revealing the association of the polytopic Na+-K+-ATPase (26, 36) and of the single transmembrane-spanning CD44 protein (37) with the cadherin-dependent ankyrin-fodrin matrix underlying the basolateral surface of polarized MDCK cells.

As shown in Fig. 2A, both the wild-type α2A-AR and the Δ3i α2A-AR are released from polarized MDCKII cells when exposed to 0.2% Triton X-100 in the presence of 2 mM EDTA and 2 mM EGTA (0 mM [Ca2+]o and 0 mM [Mg2+]o). For comparison, proteins directly associated with the cytoskeleton, such as the Na+-K+-ATPase, are not completely extracted by 0.5% Triton X-100 under similar, but slightly more stringent, divalent cation-free extracellular conditions, whereas the α2A-AR is completely extracted (26, 36), suggesting that the α2A-AR does not interact directly or stably with the cytoskeleton. When Triton X-100 extractions were performed in the presence of 0.5 mM Ca2+ and 1 mM Mg2+, the amount of Triton X-100 required for extraction of ~60% of the photoaffinity-labeled surface receptors was increased from 0.2% (Fig. 2A) to 0.5% (Fig. 2B) but with no difference in extraction efficiency between wild-type and mutant α2A-AR. These data suggest that α2A-AR stability on the basolateral surface is influenced by protein-protein interactions that involve a Ca2+–(likely cadherin–)organized sub-stratum, but that these interactions cannot explain the differences in cell surface stability of the wild-type and Δ3i loop structures, as they are extracted in a comparable manner even in the presence of Ca2+.

Specific Amino Acid Sequences Within the Third Intracellular Loop Do Not Appear to Be Required for Stabilization of the α2A-AR on the Cell Surface—If bulk size of the 3i loop is sufficient for stabilization of the α2A-AR, then a loop containing the same number of amino acids as the wild-type α2A-AR should manifest the same surface stabilization characteristics as the wild-type α2A-AR 3i loop but with very little sequence homology. The sequences of the wild-type and nonsense α2A-AR 3i loops are compared in Fig. 3. In four experiments using two different clonal cell lines expressing the α2A-AR 3i nonsense loop, the half-life of this structure was indistinguishable from that characteristic of the wild-type receptor as shown in Fig. 3.

These findings are consistent with a mechanism where the size of the 3i loop structure plays an important role in stability of the α2A-AR on the cell surface. There are examples of membrane proteins localized in surface microdomains by virtue of so-called “corrals,” often established by the cytoskeletal proteins underlying the cell surface (38–40). Consequently, α2A-AR surface stability might arise by steric principles, dictated by the size of the 3i loop (Fig. 3). If corrals partitioned the α2A-AR, then we should expect a more rapid lateral diffusion coefficient and a significantly greater half-life of this structure was indistinguishable from that characteristic of the wild-type receptor as shown in Fig. 3.

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Sustained Agonist Exposure in MDCKII Cells Does Not Decrease Receptor Density for Either Wild-type or Δ3i loop α2A-AR—One mechanism that might account for accelerated sur-

S. W. Edwards and W. J. Nelson, unpublished observations.
face turnover of the Δ3i loop α2A-AR would be enhanced agonist-elicited redistribution and subsequent down-regulation of this mutant receptor compared with the wild-type receptor. Consequently, we examined the effect of prolonged agonist exposure on steady-state α2A-AR density in MDCKII cells expressing wild-type or Δ3i loop α2A-AR. As shown in Fig. 4, treatment of MDCKII cells with 100 μM epinephrine for 24 h results in no detectable down-regulation of either the wild-type or the Δ3i loop α2A-AR. In fact, there is even a slight increase in receptor density following agonist incubation, perhaps because of ligand-dependent receptor stabilization (42). These findings are consistent with previous reports that the α2A-AR subtype does not undergo agonist-induced down-regulation in MDCKII cells (43) and Chinese hamster fibroblast cells (28), although this subtype has been reported to down-regulate in Chinese

3 M. H. Wilson and L. E. Limbird, submitted for publication.
Neither the \( \alpha_{2A}\)-AR nor the \( \Delta 3i \) loop \( \alpha_{2A}\)-AR remains associated with Triton X-100-resistant structures in polarized MDCKII cells. Polarized MDCK cells grown for 1 week on Transwell filters were radiolabeled in intact cells using 0.2 \( \mu \)Ci/well (0.9 nM) \( ^{125}\)I-Rau-AzPec, an \( \alpha_{2A}\)-AR-selective photoaffinity label. Proteins present on the basolateral surface, including the \( \alpha_{2A}\)-AR, were covalently modified with Sulfo-NHS-biotin as described under "Experimental Procedures." Wild-type or \( \Delta 3i \) loop \( \alpha_{2A}\)-AR were then extracted with increasing concentrations of Triton X-100 by rocking each Transwell in extraction buffers containing varying concentrations of Triton X-100 as indicated. All buffers contained either 2 mM EDTA, 2 mM EGTA (denoted [0 mM Ca\(^{2+}\)]\(_{o}\) and [0 mM Mg\(^{2+}\)]\(_{o}\]) or 0.5 mM Ca\(^{2+}\), 1.0 mM Mg\(^{2+}\)]\(_{o}\). After exposure to 1% Triton X-100, the residual cellular material, defined as Triton shells, was solubilized with RIPA buffer (Non Ext.). Biotinylated proteins were isolated using streptavidin-agarose chromatography. The \( \alpha_{2A}\)-AR in the eluates was resolved on a 10% SDS-polyacrylamide gel. Control experiments indicated that the radioactive band shown corresponds to \( ^{125}\)I-Rau-AzPec photoaffinity-labeled \( \alpha_{2A}\)-AR, based on its relative migration on 10% gels and the blockade of its labeling by \( \alpha_{2A}\)-AR antagonists. The results shown compare wild-type \( \alpha_{2A}\)-AR (Tag3 clone at 25 pmol/mg of protein) and \( \Delta 3i \) loop \( \alpha_{2A}\)-AR (T3 at 3.4 pmol/mg of protein (A) or T66B at 2.8 pmol/mg of protein (B)). These data are representative of at least three separate experiments. This extraction profile is not dependent on receptor density because two cell lines with nearly 10-fold different levels of wild-type \( \alpha_{2A}\)-AR expression (Tag3 clone at 25 pmol/mg of protein versus T24 clone at 3.4 pmol/mg of protein) were examined with the same results.

**CONCLUSION**

The present findings suggest that the retention of the \( \alpha_{2A}\)-AR on the basolateral surface of polarized renal epithelial cells involves the 3i loop in its entirety, because smaller deletions within the 3i loop cannot mimic the accelerated turnover characteristic of the mutant \( \Delta 3i \) loop \( \alpha_{2A}\)-AR. Stabilization of the \( \alpha_{2A}\)-AR on the basolateral surface does not appear to involve interaction with cytoskeletal proteins, because concentrations of Triton X-100 that do not destabilize direct membrane protein interaction with the cytoskeleton nonetheless extract the wild-type \( \alpha_{2A}\)-AR and \( \Delta 3i \) loop \( \alpha_{2A}\)-AR with a similar concentration-response. Deletion of the 3i loop does not accelerate agonist-elicited redistribution and down-regulation of the \( \alpha_{2A}\)-AR, suggesting that the 3i loop likely does not prevent \( \alpha_{2A}\)-AR interactions with proteins involved in receptor inter-

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\(^4\) N. L. Schramm and L. E. Limbird, submitted for publication.

\(^5\) J. R. Keefer, S. W. Edwards, and L. E. Limbird, unpublished observations.
specific amino acid sequences are not necessarily required for basolateral retention and suggesting that the bulk of the 3i loop may be sufficient to stabilize the α2A-AR on the basolateral surface.

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