Subtype-Specific Intracellular Trafficking of $\alpha_2$-Adrenergic Receptors

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SUMMARY

The three $\alpha_2$-adrenergic receptor subtypes ($\alpha_{2a}$, $\alpha_{2b}$, and $\alpha_{2c}$) are highly homologous G protein-coupled receptors. These receptors all couple to pertussis toxin-sensitive G proteins and have relatively similar pharmacological properties. To further explore functional differences between these receptors, we used immunocytochemical techniques to compare the ability of the three $\alpha_2$-receptor subtypes to undergo agonist-mediated internalization. The $\alpha_{2a}$-receptor does not internalize after agonist treatment. In contrast, we observed that the $\alpha_{2c}$-receptor is able to undergo agonist-induced internalization and seems to follow the same endosomal pathway used by the $\beta_2$-adrenergic receptor. Attempts to examine internalization of the $\alpha_{2a}$-receptor were complicated by the fact that the majority of the $\alpha_{2c}$-receptor resides in the endoplasmic reticulum and cis/medial Golgi and there is relatively little cell surface localization. Nevertheless, we were able to detect some internalization of the $\alpha_{2c}$-receptor after prolonged agonist treatment. However, we observed no significant movement of $\alpha_{2c}$-receptor from the intracellular pool to the plasma membrane during a 4-hr treatment of cells with cycloheximide, suggesting that these cells are unable to process $\alpha_{2c}$-receptors in the same way they process the $\alpha_{2a}$ or $\alpha_{2b}$ subtypes.

The $\alpha_2$-AR family consists of three highly homologous subtypes: $\alpha_{2a}$, $\alpha_{2b}$, and $\alpha_{2c}$ (1, 2). They belong to the superfamily of G protein-coupled receptors and mediate the physiological actions of the endogenous catecholamines, epinephrine and norepinephrine. These receptors are targets for therapeutic agents for hypertension, anesthesia, and analgesia. Although the three $\alpha_2$-ARs are highly homologous (50–60% identity), several differences in their coupling to G proteins α subunits and their desensitization have been reported. In reconstitution experiments, Kurose et al. (3) have shown that the human $\alpha_{2a}$ and $\alpha_{2c}$-ARs couple to the same G proteins α subunits and their desensitization have been reported. In contrast, using transfected cell lines, others (4) have shown that the $\alpha_{2c}$-subtype (the rat RG10 receptor) preferentially couples to $G_{i\alpha}$, whereas $\alpha_{2a}$ and $\alpha_{2c}$-ARs couple to $G_i$ (5). Initial studies indicated that $\alpha_2$-ARs transduce their signal through a pertussis toxin-sensitive G protein ($G_s$ or $G_o$); however, several more recent studies have demonstrated that all three $\alpha_2$-AR subtypes can stimulate adenyl cyclase in pertussis toxin-treated cells (6). The EC$_{50}$ value of agonists for stimulation of adenyl cyclase by $\alpha_{2a}$-ARs, presumably through $G_s$, is higher than the EC$_{50}$ value for inhibition of adenyl cyclase through $G_i$ (6). The three $\alpha_2$-AR subtypes seem to differ in their efficiency of coupling to $G_i$ (6). Moreover, the capacity and efficacy of $G_s$ coupling by the three $\alpha_2$-ARs are dependent on the agonist (7) and the cell line used to express the $\alpha_{2c}$-AR subtype (6, 8). Similarly, others have shown that depending on the cell line studied, $\alpha_{2a}$-ARs can inhibit or increase cellular cAMP levels, suggesting that these differences are due to coupling to different G proteins or isoforms of adenylate cyclase (9). Cotechia et al. (10) demonstrated that $\alpha_{2a}$- and $\alpha_{2c}$-ARs can stimulate phospholipase C activity. The $\alpha_{2a}$-AR has also been shown to inhibit voltage-dependent calcium currents and increase inwardly rectifying potassium currents (11); however, the ability of $\alpha_{2a}$ and $\alpha_{2c}$ to couple to these ion channels has not been reported. Finally, differences in receptor regulation have been described for the three $\alpha_2$-ARs. Agonist-promoted desensitization has been observed for all $\alpha_2$-AR subtypes; however, the extent of desensitization is greater for the $\alpha_{2a}$ subtype (12, 13). It has also been shown that phosphorylation of the $\alpha_{2a}$-AR is required for desensitization (14, 15).

In addition to these functional differences in signal transduction and regulation, recent studies have identified differ-
ences in the intracellular trafficking of this receptor family. The $\alpha_{2c}$ and $\alpha_{2b}$-ARs have been observed to reside primarily in the plasma membrane, whereas a large portion of the $\alpha_{2c}$-AR is found in an intracellular compartment (16, 17). We previously reported that there is no detectable agonist-induced internalization of the $\alpha_{2a}$-AR in cells in which rapid agonist-induced internalization of the $\beta_{2}$-AR is seen (16). Previously, agonist-induced internalization of the $\alpha_{2b}$ and $\alpha_{2c}$-AR subtypes have not been thoroughly characterized using immunocytochemical techniques; however, these receptors are structurally and functionally more similar to the $\alpha_{2a}$-AR than to the $\beta_{2}$-AR and might be predicted to have similar trafficking properties. In this study, we examined the steady state distribution and agonist-induced subcellular sorting of each of the three $\alpha_{2}$-AR subtypes in the same cell line. We observed that each subtype has distinctive trafficking properties. The $\alpha_{2c}$ and $\alpha_{2b}$-ARs are located on the plasma membrane at steady state, whereas most of the $\alpha_{2a}$-AR is found in the endoplasmic reticulum. The $\alpha_{2b}$ and $\alpha_{2c}$-AR subtypes are internalized into endosomes after exposure to agonists, whereas the $\alpha_{2a}$ subtype is not.

**Experimental Procedures**

**Materials.** [3H]Atipamezole (50 Ci/mmol) and dexamethasone were kindly provided by Orion-Farmos (Turku, Finland). All other ligands and chemicals were purchased from Sigma Chemical (St. Louis, MO) unless indicated.

**Plasmid construction and receptor expression in tissue culture cells.** All cells were grown in DMEM (University of California, San Francisco Cell Culture Facility) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA) and 25 $\mu$g/ml gentamicin (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The three mouse $\alpha_{2}$-ARs were cloned into pBC12BI, pREP4, or pcDNA3 expression vectors (Invitrogen, San Diego, CA) and epitope-tagged as previously described (16). Briefly, the 12CA5 epitope (sequence MGYPYDVPDYA) was inserted at the amino terminus of the three mouse $\alpha_{2}$-ARs, using oligonucleotide linker-adapters into the NcoI site located at the 5’-end of the receptor coding sequence. The sequence of the recombinant $\alpha_{2}$-ARs was confirmed by dideoxy sequencing. To monitor the trafficking pathway of $\alpha_{2}$-ARs between intracellular organelles and the cell surface, a double epitope tag with a thrombin cleavage site between the two epitopes was added to the amino termini of the flag epitope tag with a thrombin cleavage site between the two epitopes.

**Production of antisera.** The antigenic epitopes selected for the polyclonal antibodies were the carboxy termini of each $\alpha_{2}$-AR. The antigenic peptides were synthesized and MBS-coupled to thyroglobulin, purified, and injected into New Zealand White rabbits. Polyclonal sera directed against the $\alpha_{2a}$ and $\alpha_{2c}$-epitopes were affinity-purified over peptide coupled to epoxy-activated Sepharose columns and eluted with either 3 M KSCN, pH 7.4, or 20 mM glycine, pH 3.0. The three polyclonal antisera are specific for their respective $\alpha_{2}$-AR subtype and do not cross-react with other $\alpha_{2}$-AR subtypes at the dilutions used ($\alpha_{2a}$, 1:500; $\alpha_{2b}$, and $\alpha_{2c}$ affinity-purified antisera, 1:100; data not shown). The previously described $\beta_{2}$-antibody was kindly provided by Dr. M. Von Zastrow (University of California, San Francisco).

**Immunocytochemical studies and techniques.** Receptor trafficking and subcellular distribution were examined using indirect immunocytochemical staining. Nonspecific binding was blocked with bovine serum albumin. Each of the three $\alpha_{2}$-AR subtypes was detected by immunostaining using the appropriate monoclonal antibody and a secondary antibody conjugated to fluorescein isothiocyanate or Texas red. Studies involving basal trafficking of $\alpha_{2c}$-ARs were carried out in the absence of agonist after a 30-min treatment with 5 nM thrombin to cleave off the M1 epitope from receptors present on the plasma membrane.

For binding studies, COS-7 cells were transiently transfected using the DEAE-Dextran method, and cell membranes were harvested 3 days after transfection as previously described (21). Binding experiments were performed in 500-µl volumes of binding buffer (75 mM Tris, 12.5 mM MgCl2, and 1 mM EDTA, pH 7.4) for 90 min at room temperature. The bound radioactivity was separated from free by filtration through GF/C filters and washed three times with 5 ml of ice-cold binding buffer using a Brandel cell harvester. Saturation isotherms were performed by incubating the membranes with varying concentrations of [3H]Atipamezole. Nonspecific binding was determined by the addition of 100 $\mu$M yohimbine. Competition binding experiments were carried out by incubating membranes with varying concentrations of competing ligand and 2 $\mu$M [3H]Atipamezole. Nonspecific binding was determined by the addition of 100 $\mu$M yohimbine. Equilibrium dissociation constants were determined from saturation isotherms and competition curves using GraphPAD software (San Diego, CA). All binding experiments were done in duplicate and repeated at least three times.

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cycloheximide to inhibit de novo protein synthesis for 30 min at 37°.
Steady state trafficking of the cytosolic α₂ subunit, thrombin epitope-tagged receptor was also studied in the absence of de novo protein synthesis by treated cells with 10 μM cycloheximide for 4 hr. During inhibition of protein synthesis, cells were concomitantly treated with 5 mM thrombin to remove the M1 epitope from all receptors that were translocated to the plasma membrane.

For studies using thrombin epitope-tagged α₂-ARs, after 2 days of growth on coverslips, cells were washed three times with PBS and incubated at 37° with 6% CO₂ in DMEM with 40 mM HEPES, pH 7.4, for 30–45 min. After various treatments as outlined above, cells were rinsed three times with PBS and fixed using cold methanol (−20°) for 5 min. After fixation, cells were washed three times with PBS supplemented with Ca²⁺/Mg²⁺ over a 5-min period. To block nonspecific binding, 5% nonfat dry milk in PBS with Ca²⁺/Mg²⁺ supplemented with 50 mM HEPES, pH 7.4, was applied to cells for a period of 30–45 min. Selective dual antibody labeling of cells was performed in the blocking agent applied at room temperature for 1 hr with either the monoclonal M1 antibody (Kodak-IBI) at 1:500 or a polyclonal antibody to the hirudin epitope (Dr. S. Coughlin, University of California, San Francisco, CA) at 1:1000.

Colocalization immunocytochemical studies were performed on either the wild-type α₂, or 12CA5 epitope-tagged α₂-AR and a variety of antibodies selective to various intracellular compartments. Cells were costained with either the 12CA5 monoclonal antibody or a polyclonal antibody to the hirudin epitope (Dr. S. Coughlin, University of California, San Francisco, CA) at 1:500. After primary antibody labeling, the cells were counterstained with fluorescein isothiocyanate or Texas red imaging. In nonpermeabilized cells at steady state, there is a relatively small amount of the α₂c-AR present in the plasma membrane (data not shown). F, A 30-min incubation with 10 μM norepinephrine did not cause noticeable redistribution of the α₂c-AR.

**TABLE 1**

<table>
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<th></th>
<th>α₂a</th>
<th>12CA5-α₂a</th>
<th>α₂b</th>
<th>12CA5-α₂b</th>
<th>α₂c</th>
<th>12CA5-α₂c</th>
<th>Thrombin-α₂c</th>
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<td>1454 ± 434</td>
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<td>(−)-Norepinephrineb</td>
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<td>3349 ± 1013</td>
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<td>484 ± 128</td>
<td>648 ± 90</td>
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<td>Dexamethasone</td>
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<td>16.4 ± 0.6</td>
<td>7.2 ± 0.4</td>
<td>8.6 ± 4.5</td>
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* Kᵣ  
b Kᵦ  
N.D., not determined.

**Fig. 1.** Distribution of the three α₂-AR subtypes in permeabilized HEK 293 cells at steady state and after agonist treatment. Agonist treatment, cell fixation, permeabilization, and staining were described in Experimental Procedures. A, At steady state, the α₂a-AR was primarily localized to the plasma membrane. B, After a 30-min incubation with 10 μM norepinephrine, the α₂a-AR did not redistribute. C, Similar to the α₂a at steady state the α₂b-AR was primarily localized in the plasma membrane. D, However, 10 μM norepinephrine treatment caused the α₂b-AR to internalize into intracellular vesicles. E, In contrast to α₂a and α₂b-AR sorting at steady state, the α₂c-AR appears as punctate intracellular staining. In nonpermeabilized cells at steady state, there is a relatively small amount of the α₂c-AR present in the plasma membrane (data not shown). F, A 30-min incubation with 10 μM norepinephrine did not cause noticeable redistribution of the α₂c-AR.
phosphatase (BioRad, Richmond, CA) diluted 1:1000 in 1% BSA for 60 min with occasional gentle shaking. Wells were then washed three times with PBS, and a colorimetric alkaline phosphate substrate was added. Plates were continuously but gently shaken (40 rotations/min) until an adequate color change had occurred, at which time a 100-μl sample was taken for colorimetric readings. Nontransfected cells were studied concurrently to determine background. All experiments were done in triplicate.

Results

Immunocytochemical techniques were used to examine the distribution of α2-AR subtypes expressed in COS 7, HEK 293, MDCK, NRK and Rat1 fibroblast cell lines. Subtype-specific rabbit polyclonal antibodies were prepared against a carboxyl-terminal peptide as described in Experimental Procedures. The specificity of these antibodies was verified by staining untransfected COS 7 cells or COS 7 cells transfected with each of the α2-AR subtypes (data not shown). In addition, we used receptors modified with an amino-terminal 12CA5 epitope, which is recognized by a commercially available monoclonal antibody. The amino-terminal tag permits examination of cell surface receptor density in nonpermeabilized cells. We previously observed that the use of this epitope does not alter the trafficking of the β2-AR (16). Moreover, the distribution and trafficking of epitope tagged α2-ARs (examined with either 12CA5 monoclonal antibody or polyclonal subtype-specific antibody) are indistinguishable from those of nontagged receptors (examined with polyclonal antibody; data not shown). Binding affinity for the antagonist atipamezole, the endogenous catecholamines epinephrine and norepinephrine, and the specific α2 agonist dexmedetomidine are also comparable between wild-type and epitope-tagged α2-ARs (Table 1).

Fig. 1 shows the distribution of the three α2-AR subtypes in HEK 293 cells at steady state and after agonist treatment. It can be seen that α2a and α2b subtypes are predominantly localized in the plasma membrane at steady state, whereas a large portion of α2c-AR is found in an intracellular compartment. After agonist treatment (10 μM norepinephrine) for 30 min at 37°C, the α2c-AR is internalized, but the α2a subtype

Fig. 2. Receptor distribution after agonist treatment in HEK 293 cells coexpressing α2a- and α2b-ARs. The 12CA5 epitope-tagged α2b-AR was transiently transfected into a nonclonal HEK 293 cell line stably expressing the α2a-AR. After treatment, cells were fixed and permeabilized as described in Experimental Procedures. At steady state, receptor was localized to the plasma membrane in cells expressing both (A) α2a- and (B) α2b-AR subtypes. C, After a 30-min incubation with 10 μM norepinephrine, the α2a-AR did not redistribute. D, In contrast, agonist treatment caused the α2b-AR coexpressed in the same cell to internalize into endosomes. Similar treatment with a 10 nm concentration of the nonselective α2 agonist dexmedetomidine also selectively internalized the α2b-AR (F), whereas no α2a-AR internalization could be detected in the same cells (E).

Fig. 3. Effect of subtype-selective and -nonselective agonist treatment of HEK 293 cells coexpressing α2a- and β2-ARs. The 12CA5 epitope-tagged mouse α2b-AR was transiently cotransfected with the human β2-AR in HEK 293 cells. At ~24 hr after transient transfection, cells were plated onto glass coverslips and grown for 2 days. For treatments, coverslips were incubated for 30 min in serum-free DMEM (control) or with the appropriate agonist in serum-free DMEM at 37°C and 6% CO2. After fixation, cells were permeabilized before staining. A and B. At steady state, both receptors are localized in the plasma membrane. In cells coexpressing both ARs, 10 μM isoproterenol, a β-selective agonist, induced internalization of β2-ARs (D), whereas α2b-ARs did not redistribute (C). Similarly, 10 nm dexmedetomidine, an α2-selective agonist, promoted internalization of α2b-ARs (E), whereas β2-ARs did not redistribute (F). When cells coexpressing both α2a- and β2-ARs were treated with 10 μM norepinephrine, a common agonist for α2- and β2-ARs, both receptors internalized into the same endocytic vesicles as observed by confocal microscopy (G and H, respectively).
remains in the plasma membrane. This analysis does not allow us to determine whether the \( \alpha_2c\)-AR is internalized because of the large background of \( \alpha_2\)-AR already in an intracellular membrane compartment. This intracellular pool of \( \alpha_2c\)-AR is further characterized below.

To further investigate differences in agonist-mediated internalization, 12CA5 epitope-tagged \( \alpha_{2b}\)-AR was coexpressed with a nontagged \( \alpha_{2a}\)-AR, allowing us to examine differences in trafficking between these two subtypes in the same cell. The \( \alpha_{2a}\) distribution was monitored with the polyclonal \( \alpha_{2a}\) subtype-specific antibody, and the epitope-tagged \( \alpha_{2b}\)-AR distribution was monitored with a monoclonal antibody to the 12CA5 epitope. At steady state, both receptors are found in the plasma membrane of the same cell (Fig. 2, A and B). After exposure to 10 \( \mu M \) norepinephrine for 30 min, only the \( \alpha_{2b}\)-AR is internalized (Fig. 2, C and D). Similar treatment with 10 \( \mu M \) of the nonselective \( \alpha_2\) agonist dexmedetomidine also selectively internalized the \( \alpha_{2b}\)-AR in cotransfected cells (Fig. 2, E and F). Additional agonist treatment paradigms with the \( \alpha_{2a}\)-AR (30-min incubations with 10 \( \mu M \) epinephrine or 100 \( \mu M \) norepinephrine or \( \leq 4\)-hr incubation with 10 \( \mu M \) norepinephrine) resulted in similar findings (data not shown).

The \( \alpha_{2a}\) and \( \beta_2\)-ARs internalize by a similar mechanism. The mechanism of agonist-mediated internalization of the \( \beta_2\)-AR has been well characterized. We therefore coexpressed the epitope-tagged \( \alpha_{2a}\)-AR along with the \( \beta_2\)-AR and examined the distribution of the two receptors after agonist treatment. At steady state, both receptors are on the plasma membrane (Fig. 3, A and B). Only the \( \beta_2\)-AR is internalized in cells exposed to the \( \beta_2\)-AR agonist isoproterenol (Fig. 3, C and D), and only the \( \alpha_{2b}\)-AR is internalized in cells exposed to the \( \alpha_2\)-AR agonist dexmedetomidine (Fig. 3, E and F). Using confocal microscopy, we demonstrated that both receptors are internalized by exposure to the common agonist norepinephrine and seem to reside in the same endocytic vesicles (Fig. 3, G and H).
The results of these immunocytochemical studies suggest that there is no internalization of the \(\alpha_{2a}\)-AR after agonist treatment; however, it may not be possible to detect internalized receptor by fluorescence microscopy if it is not concentrated in endosomes. To investigate this possibility, we used an ELISA to monitor the loss of cell surface receptor rather than accumulation of intracellular receptor protein after agonist activation. Epitope-tagged \(\alpha_{2a}\)-and \(\alpha_{2b}\)-ARs were expressed in HEK 293 cells, and the density of receptors was monitored in nonpermeabilized cells with the 12CA5 antibody. At steady state, the cell surface expression of the \(\alpha_{2a}\)- and \(\alpha_{2b}\)-ARs was comparable. After treatment with noradrenaline for 30 min, there was a loss of \(31 \pm 2.4\%\) (mean \(\pm\) standard error) of \(\alpha_{2b}\)-ARs and a loss of \(7 \pm 1.4\%\) of \(\alpha_{2a}\)-ARs. These results confirm the difference between \(\alpha_{2a}\)- and \(\alpha_{2b}\)-ARs; however, they suggest that there is a small but significant agonist-induced internalization of the \(\alpha_{2a}\)-AR, possibly by a nonendosomal mechanism.

The \(\alpha_{2a}\)-AR is found in the endoplasmic reticulum in Rat1 fibroblast cells. Although there is some plasma staining, the \(\alpha_{2c}\)-AR seems to reside primarily in an intracellular compartment in HEK 293 cells (Fig. 1C). To further study this, we expressed the \(\alpha_{2a}\)- and \(\alpha_{2b}\)-ARs in a variety of cell lines. Similar to studies reported by Wozniak et al. (17) when expressed in a MDCK II cell line, the abundance of \(\alpha_{2a}\)-AR was localized in an intracellular compartment, whereas the \(\alpha_{2a}\)-AR was targeted to the basolateral plasma membrane (Fig. 4, A and B). When the 12CA5 epitope-tagged \(\alpha_{2a}\)-AR was transiently cotransfected in an HEK-293 cell line stably expressing wild-type \(\alpha_{2a}\)-AR, the \(\alpha_{2c}\) subtype was also localized to an intracellular compartment (Fig. 4, C and D). Co-expression of the \(\alpha_{2c}\) did not alter the trafficking of the \(\alpha_{2a}\)-AR subtype. We attempted to quantify the amount of 12CA5 epitope-tagged \(\alpha_{2a}\)-AR in the plasma membrane using the ELISA method discussed above. In contrast to the \(\alpha_{2a}\)- and \(\alpha_{2b}\)-AR subtypes, the signal generated from \(\alpha_{2c}\)-AR transfected cells was not significantly different from that of untransfected cells. Thus, although we were able to detect a small amount of 12CA5 epitope-tagged \(\alpha_{2c}\)-AR in the plasma membrane of nonpermeabilized cells by the sensitive technique of immunofluorescence microscopy, the amount was too small to quantify by ELISA.

The identity of the intracellular compartment containing the \(\alpha_{2c}\)-ARs was difficult to assess in HEK 293 cells because of their relatively small size. To further investigate the subcellular distribution of \(\alpha_{2c}\)-ARs, we studied Rat1 fibroblast and NRK cells, which have relatively large cytoplasmic compartments. The distribution of the receptor in Rat1 cells colocalizes with both BiP and mannosidase II (Fig. 5, A–D), suggesting that the \(\alpha_{2a}\)-AR is found primarily in the endoplasmic reticulum and cis/medial Golgi. No overlap was observed with markers for trans-Golgi network (M6PR), endosomes (M6PR), or lysosomes (lgp120) (Fig. 5, E–H). We observed a similar distribution of the \(\alpha_{2c}\)-AR in NRK cells (data not shown).

The finding that a large proportion of cellular \(\alpha_{2c}\)-AR resides in the endoplasmic reticulum suggests several possibilities. This receptor may be improperly processed, or the cell may lack an important chaperone protein and therefore the receptor is retained in the endoplasmic reticulum. It is also possible that the receptor is processed slowly and undergoes a relatively rapid turnover at the plasma membrane. However, similar half-lives have been reported for all three \(\alpha_{2}\)-AR subtypes in MDCK cells (17). Finally, it is possible that the receptor is cycling between the plasma membrane and cytosolic pool. To examine these possibilities, we constructed a modified form of the \(\alpha_{2c}\)-AR that would allow us to identify receptor that has been delivered to the plasma membrane. Fig. 6 shows the amino-terminal sequence of the modified \(\alpha_{2c}\)-AR. An M1 flag epitope and a thrombin cleavage site were added to the amino terminus. Cleavage of the receptor by thrombin present in the media results in loss of the flag epitope (which can be recognized by the commercially available M1 monoclonal antibody), whereas the hirudin binding domain of the thrombin cleavage site remains and can be recognized by a polyclonal antibody. Therefore, receptors that are on the plasma membrane are susceptible to thrombin cleavage. These thrombin-cleaved receptors will stain only with the polyclonal antibody to the hirudin epitope, not with the M1 flag antibody.

Fig. 7 demonstrates that the amino-terminal thrombin
epitope does not effect the distribution of the $\alpha_2$-AR. In permeabilized cells, most of the $\alpha_2$-AR is found in an intracellular compartment (Fig. 7, E–H); however, cell surface expression can be demonstrated in nonpermeabilized cells (Fig. 7, A–D). Exposure of the cell to thrombin results in loss of cell surface receptor staining by the M1 flag antibody (Fig. 7, C and G); however, the receptor is still recognized by the antibody to the hirudin epitope (Fig. 7, D and H). To investigate the turnover of $\alpha_2$-AR, cells were exposed to cycloheximide to block protein synthesis and incubated in the presence of epinephrine and thrombin for 4 hr (Fig. 8, A–F). Movement of receptor from the intracellular pool to the cell surface would result in cleavage by thrombin and a loss of staining by M1 flag antibody. If these receptors were then internalized and recycled through the endoplasmic reticulum or distributed to lysosomes, they would be recognized by staining with the polyclonal antibody to the hirudin epitope but not the M1 flag antibody. As seen in Fig. 8, there is no significant loss of intracellular staining by the M1 antibody, indicating that the intracellular pool of receptor is stable. Moreover, using the cleavable thrombin/flag epitope, we were able to determine that a small amount of $\alpha_2$-AR was internalized from the plasma membrane with agonist treatment (Fig. 8, E and F). This internalized receptor stains with the hirudin antibody but not the M1 antibody (Fig. 8, E and F, arrows).

In a comparable experiment, the $\alpha_2$-AR was similarly tagged with the same thrombin-cleavable epitope (Fig. 9). At steady state, most of the receptor is susceptible to cleavage with thrombin, indicating that the majority of $\alpha_2$-AR is localized in the plasma membrane (Fig. 9, C and D). A small amount of intracellular receptor (M1 flag antibody staining) is observed before but not after exposure to cycloheximide and thrombin for 4 hr (Fig. 9, E and F). This indicates that within 4 hr, all of the $\alpha_2$-AR in the biosynthetic pathway has been processed and inserted in the plasma membrane.

Agonist-promoted accumulation of $\alpha_2$-AR in endosomes may not be as pronounced as that observed for the $\alpha_2$-AR because of the large amount of $\alpha_2$-AR in the intracellular compartment at steady state and the comparatively small amount of $\alpha_2$-AR in the plasma membrane (Fig. 8F). A more sensitive technique for identifying agonist-induced receptor internalization is shown in Fig. 10. Living, nonpermeabilized cells expressing M1 flag-tagged $\alpha_2$-AR are first labeled with M1 flag monoclonal antibody and then washed and incubated in the presence or absence of agonist for 30 min. Cells were then fixed, permeabilized, and stained with secondary antibody. This approach allowed us to selectively examine only $\alpha_2$-ARs that are in the plasma membrane. No internalization was detected in control cells (Fig. 10A); however, after agonist treatment, receptor was observed in an intracellular compartment (Fig. 10C, arrow). The intracellular receptor surrounds the nucleus, creating a nuclear shadow in agonist-treated cells (Fig. 10C), whereas no nuclear shadow is observed in control cells (Fig. 10A). The internalized $\alpha_2$-AR colocalized with the M6PR that is present in the trans-Golgi network and endosomes (Fig. 10D).

**Discussion**

In this study, we report differences in steady state targeting and agonist-induced internalization of the three $\alpha_2$-AR subtypes. The $\alpha_{2a}$-AR behaves more like the $\beta_2$-AR than the $\alpha_2$-AR. This is particularly interesting in light of the high degree of identity shared by $\alpha_{2a}$ and $\alpha_{2b}$ (55%, $\alpha_{2a}$ versus $\alpha_{2b}$; 21%, $\beta_2$ versus $\alpha_{2b}$). Moreover, we extend previous observations regarding the intracellular distribution of the $\alpha_2$-AR subtype. The roles of intracellular targeting and trafficking in signal transduction are not well understood; however, agonist-induced internalization has been shown to play a role in receptor regulation. Studies have implicated agonist-induced internalization in the process of resensitization. Blocking internalization by selective receptor mutations or treatment of cells with concanavalin A or sucrose prevents dephosphorylation of the receptor (22). Recent studies have suggested that binding of $\beta$-arrestin to the $\beta_2$-AR after G protein-coupled receptor kinase phosphorylation is necessary for internalization (23). Of interest, the $\alpha_{2a}$-AR has been...
Shown to undergo phosphorylation by G protein-coupled receptor kinase (14, 15). The inefficient agonist-induced internalization in the $\alpha_{2a}$-AR may suggest that this receptor does not bind to $\beta$-arrestin. These results may also suggest that the $\alpha_{2b}$-AR is tethered to the plasma membrane and is prevented from undergoing internalization even when phosphorylated and bound to $\beta$-arrestin. This may lead to a difference in the rate of resensitization and therefore account for the more extensive desensitization observed for the $\alpha_{2a}$-AR.

We observed a small amount of agonist-induced internalization of $\alpha_{2a}$ using a sensitive ELISA technique for quantitative changes in cell surface density of receptor antigen. However, there was no significant accumulation of receptor in endosomes that could be detected by immunocytochemistry. This suggests that the small amount of $\alpha_{2a}$ internalization may be occurring by a different mechanism than that used for internalization of $\alpha_{2b}$- and $\beta$-ARs. The inefficient agonist-induced internalization of $\alpha_{2a}$ relative to $\alpha_{2b}$ is in contrast to previous reports (12, 24); Eason et al. found that a 30-min exposure to agonist resulted in 35% sequestration of the $\alpha_{2a}$-AR and 26% sequestration in the $\alpha_{2b}$-AR. In those studies, CHO cells were used, and internalization was assayed by ligand binding techniques that used hydrophilic agonists to distinguish between cell surface and intracellular receptor. Several possibilities might account for these different results. First, CHO cells may express other proteins that are critical for agonist-induced sequestration in the $\alpha_{2a}$-AR.
Hirudin staining in the plasma membrane is retained (E). After norepinephrine for 4 hr, there is a complete loss of flag staining (F), whereas cells that have been treated with thrombin, cycloheximide, and norepinephrine for 4 hr. After fixation, immunocytochemical staining was performed after cellular permeabilization as described in Experimental Procedures. Similar to wild-type or 12CA5 epitope-tagged $\alpha_2$-ARs, at steady state, thrombin/flag epitope-tagged receptor is primarily localized to the plasma membrane (A and B). Thrombin treatment caused a complete loss of flag staining in the plasma membrane (D), whereas hirudin staining was retained (C). In cells that have been treated with thrombin, cycloheximide, and norepinephrine for 4 hr, there is a complete loss of flag staining (F), whereas hirudin staining in the plasma membrane is retained (E).

Although $\alpha_2$- and $\beta_2$-ARs undergo agonist-promoted receptor internalization in HEK 293 cells, it is possible that this cell line lacks a component necessary for sequestration of $\alpha_2$-ARs that is present in CHO cells. Another possible explanation is that different methods were used to assess sequestration in the two studies. In the current study, we used immunocytochemical methods to examine receptor internalization. These techniques are not influenced by the state of receptor/G protein coupling. This may not be the case when $\alpha_2$-AR internalization is examined by using a hydrophilic agonist to quantify cell surface receptor density. It is possible that agonist treatment could reduce agonist binding of desensitized plasma membrane $\alpha_2$-AR even in the absence of receptor internalization.

The physiological or functional significance of the large intracellular pool of the $\alpha_2$-AR subtype is not known at this time. Both we and others have reported this unique intracellular distribution in a variety of cell lines, including COS-7, HEK 293, MDCK II, Rat1 fibroblasts, and NRK (16, 17). Moreover, this subcellular distribution is not a species-specific idiosyncrasy to the mouse $\alpha_2$-AR because the wild-type human receptor shows similar intracellular localization when transfected in COS-7 or HEK-293 cells (data not shown). Wozniak and Limbird (17) have shown that in MDCK II cells, this distribution is independent of receptor expression levels; consistent with our findings that a relatively small proportion of the $\alpha_2$-AR is targeted to the plasma membrane (Fig. 4, B–D), the authors showed that this receptor is targeted directly to the basolateral membrane of MDCK II cells. It is also noteworthy that when $\alpha_2$- and $\alpha_2$-ARs are coexpressed in the same cell, these differences in receptor sorting are maintained (Fig. 4, C and D). The $\alpha_2$-AR may require a specific chaperone protein for efficient targeting to the plasma membrane, or it may require a specific protein that anchors the receptor to the cytoskeleton. In the latter case, one would expect the receptor to traffic normally to the plasma membrane but not be retained. However, this mechanism is not consistent with the results of experiments using the thrombin/flag epitope-tagged receptor (Fig. 8) that demonstrate no detectable cycling of receptor between intracellular compartment and the plasma membrane.

We noted that plasma membrane $\alpha_2$-AR was more easily visualized in NRK cells (Figs. 7, 8, and 10) than in HEK 293 cells. It may be that NRK cells are somewhat better in translocating the $\alpha_2$-AR to the plasma membrane than are HEK 293 cells; however, this may also be due to the fact that the NRK cells are larger and very flat, permitting better visualization of a larger amount of plasma membrane in a single plane of focus.

We were unable to detect agonist-induced internalization of the $\alpha_2$-AR using conventional immunocytochemical techniques (Fig. 1). This is in part due to the large preexisting pool of intracellular $\alpha_2$-AR. However, using a cleavable

**Fig. 9.** Trafficking studies of the amino-terminal thrombin/flag epitope-tagged $\alpha_2$-AR indicates that within 4-hr, all receptor has been processed and inserted into the plasma membrane. Intact NRK cells transfected with the thrombin/flag epitope-tagged $\alpha_2$-AR were grown on coverslips and incubated in (A and B, Control) DMEM, (C and D) 5 nM thrombin in DMEM for 5 min, or (E and F) 5 nM thrombin, 10 $\mu$M cycloheximide, and 10 $\mu$M norepinephrine for 4 hr. After fixation, immunocytochemical staining was performed after cellular permeabilization as described in Experimental Procedures. Similar to wild-type or 12CA5 epitope-tagged $\alpha_2$-ARs, at steady state, thrombin/flag epitope-tagged receptor is primarily localized to the plasma membrane (A and B). Thrombin treatment caused a complete loss of flag staining in the plasma membrane (D), whereas hirudin staining was retained (C). In cells that have been treated with thrombin, cycloheximide, and norepinephrine for 4 hr, there is a complete loss of flag staining (F), whereas hirudin staining in the plasma membrane is retained (E).

**Fig. 10.** Immunocytochemical identification of agonist-induced redistribution of $\alpha_2$-ARs into endosomes. Thrombin/flag epitope-tagged $\alpha_2$-ARs were stably expressed in NRK cells and grown on coverslips as described in Experimental Procedures. Coverslips were incubated with monoclonal flag antibody at 4° for 60 min, labeling epitope-tagged $\alpha_2$-ARs present on the cell surface. After gentle washing, coverslips were returned to 37° with 6% CO$_2$ and incubated in serum-free DMEM with monoclonal flag antibody at 4° for 60 min, labeling epitope-tagged $\alpha_2$-ARs present on the cell surface. After gentle washing, coverslips were returned to 37° with 6% CO$_2$ and incubated in serum-free DMEM with monoclonal flag antibody at 4° for 60 min, labeling epitope-tagged $\alpha_2$-ARs present on the cell surface.
thrombin/flag epitope on the amino terminus of the α2a-AR allowed us to identify that some receptor is internalized with prolonged agonist treatment (Fig. 8, E and F). This was confirmed by prelabeling the amino-terminal M1 flag epitope of plasma membrane α2a-AR with antibody before agonist treatment (Fig. 10).

The differences in intracellular trafficking observed for the α2-AR subtypes is somewhat unexpected considering the high degree of amino acid identity and the functional similarity with respect to ligand-binding properties and G protein coupling. The functional significance of differences in receptor trafficking is unknown but may be more important in vivo in highly differentiated cells. It has been proposed that receptor subtypes may be targeted to specific plasma membrane microdomains in which receptors are found in close proximity with specific G proteins and effector enzymes (25). Thus, unique trafficking behavior of receptors may enable receptors to couple to specific effector systems.

Summary. We reported that in several cell lines, the three α2-ARS display unique patterns of subcellular distribution and sorting. At steady state, the α2a and α2b-AR subtypes are localized in the plasma membrane. Agonist treatment induces internalization of the α2b-AR into endosomes, presumably using the same cellular machinery as the β2-AR, whereas no accumulation of α2a-AR in endosomes could be detected after agonist stimulation. In contrast, at steady state the α2a-AR is targeted to the plasma membrane; however, a significant proportion of this receptor is localized in an intracellular pool that has not yet been delivered to the plasma membrane. Through colocalization studies, we determined that the intracellular pool of α2a-ARs is predominantly localized in the endoplasmic reticulum and cis/medial Golgi. Agonist treatment of the α2a-AR results in some receptor internalization from the plasma membrane. The functional significance of the observed differences in subcellular sorting of the three relatively highly homologous α2-ARs remains to be established.

References