Rapid Agonist-induced Desensitization and Internalization of the A2B Adenosine Receptor Is Mediated by a Serine Residue Close to the COOH Terminus*

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The A2B-coupled rat A2B adenosine receptor (A2B-AR) was epitope-tagged at the NH2 terminus with hemagglutinin (HA) and subjected to progressive deletions or point mutations of the COOH terminus in order to determine regions of the receptor that contribute to agonist-induced desensitization and internalization. When expressed stably in Chinese hamster ovary cells, a mutant receptor in which the final 2 amino acids were deleted, the Leu330-stop mutant, underwent rapid agonist-induced desensitization and internalization as did the wild type (WT) receptor. However, the Phe328 and the Gln325-stop mutants were resistant to rapid agonist-induced desensitization and internalization. Co-expression of arrestin-2-green fluorescent protein (arrestin-2-GFP) with WT receptor or Leu330-stop mutant resulted in rapid translocation of arrestin-2-GFP from cytosol to membrane upon agonist addition. On the other hand, agonist activation of the Phe328-stop or Gln325-stop mutant did not result in translocation of arrestin-2-GFP from cytosol. A COOH terminus point mutant, S329G, was also unable to undergo rapid agonist-induced desensitization and internalization, indicating that Ser329 is a critical residue for these processes. A further deletion mutant (Ser326-stop) unexpectedly underwent rapid agonist-induced desensitization and internalization. However, activation of this mutant did not promote translocation of arrestin-2-GFP from cytosol to membrane. In addition, whereas WT receptor internalization was markedly inhibited by co-expression of dominant negative mutants of arrestin-2 (arrestin-2-(319–418)), dynamin (dynamin K44A), or Eps-15 (Eps-15/H900495–295), Ser326-stop receptor internalization was only inhibited by dominant negative mutant dynamin. Taken together these results indicate that Ser326, close to the COOH terminus of the rat A2B-AR, is critical for the rapid agonist-induced desensitization and internalization of the receptor. However, deletion of the COOH terminus also uncovers a motif that is able to redirect internalization of the receptor to an arrestin- and clathrin-independent pathway.

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Prolonged or repeated exposure of G protein-coupled receptors (GPCRs)1 to agonist usually results in a decrease in subsequent receptor responsiveness, a process termed desensitization (1). The mechanisms underlying rapid desensitization often involve GPCR phosphorylation by a family of G protein-coupled receptor kinases (GRKs; Ref. 2). Receptor phosphorylation by GRKs promotes binding of arrestins (3), which triggers desensitization by uncoupling the receptor from G-protein (4). Following desensitization, many GPCRs appear to internalize by an arrestin-dependent process via clathrin-coated pits (5). In most cases this leads to the eventual intracellular dephosphorylation of the receptor, and its reinsertion into the cell membrane in a resensitized state (6). However, in some cases GPCR internalization appears to contribute to desensitization (7). More prolonged agonist activation generally leads to the redirection of internalized receptor to a lysosomal compartment with subsequent down-regulation (8), although some GPCRs appear to be targeted for down-regulation after relatively short agonist treatment times (9). Thus although a general picture is emerging of how GPCR responsiveness is regulated in the presence of prolonged agonist treatment (1–4), the molecular signals that determine the pathways of desensitization, internalization, resensitization, or down-regulation remain to be clarified.

The endogenous nucleoside adenosine is now known to regulate cellular function via activation of four adenosine GPCRs, the A1, A2A, A2B, and A3-ARs (10). Both the A2A- and A2B-adenosine receptors are G coupling and stimulate cAMP formation, and the A2B-AR subtype is thought to regulate such diverse processes as vascular tone, neurosecretion, and mast cell activation (11). Agonist-induced desensitization of A2B-ARs has been reported in a number of cell types (12–16) and tissues (17). Using a combination of molecular biological techniques we have shown that the mechanism of A2B-AR desensitization likely involves GRK2 and non-visual arrestins. Thus, expression of a dominant negative mutant GRK2 construct in NG108-15 cells (12) or the antisense-induced reduction of non-visual arrestin levels in HEK293 cells (15) reduced the rate of agonist-induced desensitization of endogenous A2B-AR responsiveness very recently, we have shown that agonist activation

1 The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; CHO cells, Chinese hamster ovary cells; HA, hemagglutinin; NECA, 5′-(N-ethylcarboxamido)adenosine; Ro 201724, 4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one; WT, wild type; arrestin-2-GFP, arrestin-2-green fluorescent protein; A2AR, A2B-adenosine receptor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, sodium polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.
of A_{2B}-ARs transiently expressed in HEK293 cells promotes arrestin-2-GFP translocation from cytosol to cell membrane (18). Furthermore, the A_{2B}-ARs subsequently undergo internalization to compartments which co-localize with the endosomal markers transferrin and rab-5 (18), as well as with arrestin-2-GFP itself. Another recent study (19) indicates that the A_{2B}-AR internalizes in an arrestin- and dynamin-sensitive fashion. Together, these results indicate that A_{2B}-AR undergoes desensitization and internalization in a GRK- and arrestin-dependent manner. For other GPCRs, the COOH terminus of the receptor is often implicated as a site for GRK-mediated phosphorylation and arrestin interaction (2). For example, mutation of a threonine residue in the COOH terminus of the canine A_{2A}-AR inhibited the agonist-induced phosphorylation and desensitization of this receptor (20). At present nothing is known of the molecular determinants of the desensitization and internalization of the A_{2B}-AR. In the present study we investigated the role of the COOH terminus of this receptor in these processes using a series of COOH terminus deletion and point mutants. The results indicate that a serine residue close to the end of the COOH terminus of A_{2B}-AR is critical for rapid agonist-induced desensitization and internalization of the receptor. Furthermore, we find that altering the COOH terminus can alter the arrestin dependence of agonist-induced receptor internalization.

**EXPERIMENTAL PROCEDURES**

**Materials**—The pcDNA3 and pEGFPN1 vectors were obtained from Invitrogen and Clontech, respectively. The construct EGFP-C2-Eps15 (EJ95–295) was a kind gift from Dr. A. Benmerah. Fugene-6 and restriction enzymes were obtained from Roche Molecular Biochemicals, ligase from New England Biolabs, and DNA preparation kits from Qiagen. DMEM and DMEM/F-12 (50:50) were from Life Technologies, fetal calf serum from Harlan Sera Labs, and genetin (G418 sulfate) from Calbiochem. The anti-HA monoclonal HA.11 antibody was from BabCO, the goat anti-mouse secondary antibody conjugated with alkaline phosphatase from Sigma, the rhodamine-conjugated mouse monoclonal antibody 12CA5 from Roche Molecular Biochemicals, and the goat anti-mouse conjugated with alkaline phosphatase from Sigma. The pcDNA3-dynamin-K44A, EGFP-C2-Eps15 (E11032) and EGFP-C2-Eps15 (E11032) antisense primer 5'-TGACTCTAGATCACAAGCTCAGACTGAAAGT-3' was from Bio-Rad. [3H]cAMP was obtained from Du Pont. [3H]cAMP was obtained from Du Pont.

**Cloning, Expression, and Characterization of Constructs**—CHO-K1 cells were cultured in DMEM/F-12 (50:50) medium, 10% fetal calf serum, 100 units ml^-1 penicillin, and 100 µg ml^-1 streptomycin. Stably transfected CHO cells were cultured in the above medium supplemented with 600 µg ml^-1 geneticin. For stable transfections, 60–80% confluent CHO cells in 2 ml of medium in a 60-mm culture dish were transfected with 1 µg of DNA linearized with BgIII, using 3 µl of Fugene-6, according to the manufacturers instructions. After 24 h, cells were split (1:20) into medium containing 600 µg ml^-1 geneticin. Individual cell clones were allowed to develop for 1–2 weeks and transferred to 24-well dishes using a pipette tip. Clones functionally expressing receptor constructs were identified by measuring 10 µM NECA-stimulated cAMP accumulation, as described below.

**Immunofluorescence Microscopy and Single Cell Imaging**—To assess the cellular distribution of WT and mutant A_{2B}-AR, CHO cells stably
transfected with these constructs were grown in 6-well plates on coverslips. Cells were then incubated with primary antibody (anti-HA monoclonal, 1:1000 dilution) for 1 h at 4 °C in DMEM supplemented with 1% BSA. Cells were washed twice with PBS and then incubated at 37 °C for NECA (10 μM; 30 min) in DMEM with 0.5% BSA. The cells were then fixed with 3.7% formaldehyde/PBS for 15 min at room temperature. Nonspecific binding was blocked with bovine serum albumin (0.05% Triton X-100/PBS containing 5% nonfat dry milk) for 30 min at 37 °C. Goat anti-mouse rhodamine-conjugated secondary antibody was then added at a dilution of 1:150 in blotto for 30 min at 37 °C. The cells were then washed six times with 0.05% Triton X-100/PBS and the last wash left for 37 °C for 30 min. Finally the cells were fixed again with 3.7% formaldehyde as described. Coverslips were mounted using Slow-Fade mounting medium and examined by microscopy on an inverted Leica TCS-NT confocal laser scanning microscope attached to a Leica DM IRBE epifluorescence microscope with phase-contrast and a Plan-Apo 1.40 NA oil immersion objective. All images were collected on Leica TCS-NT software for two- and three-dimensional image analysis and processed on Adobe Photoshop 5.5.

**RESULTS**

The wild type (WT) rat A2B-AR and various COOH terminus deletion mutants (Fig. 1) were constructed by reverse transcriptase-polymerase chain reaction and epitope tagged at the NH2 terminus with a HA sequence, as described under "Experimental Procedures." These constructs were then stably transfected into CHO cells, however, we were unable to obtain CHO cells stably expressing a functional Tyr299-stop receptor mutant. The ability of the adenosine receptor agonist NECA to stimulate cAMP formation in the cells was then determined (Fig. 2A). In CHO cells stably expressing WT receptor as well as those expressing COOH terminus deletions, NECA stimulated cAMP formation with an EC50 of between 0.25 and 0.64 μM, although there was variation in the maximum response observed. CHO cells stably transfected with the pcDNA3 plasmid vector alone did not respond to NECA (Fig. 2A). Initial experiments to label A2B-AR with the A2-AR radioligand [3H]ZM 241385 (24) failed to detect specific binding in membranes prepared from CHO cells stably transfected with WT A2B-AR, under conditions where specific binding to membranes prepared from CHO cells stably transfected with WT A2A-AR was readily observed (data not shown). Therefore, in the ab-
sence of a suitable A2B-AR radioligand, an estimation of the relative expression level of WT and deletion mutant A2B-AR was obtained using an ELISA assay (23), taking advantage of the HA-epitope tag contained in the NH2 terminus of the receptor constructs. This analysis indicated that in the absence of agonist stimulation there was little difference in cell surface expression of these constructs (arbitrary absorbance units mg−1 cell protein: WT 1.37 ± 0.19; Leu330-stop 1.12 ± 0.15; Phe328-stop 1.19 ± 0.14; Ser326-stop 1.32 ± 0.12; Gln325-stop 1.07 ± 0.17; n = three separate experiments in each case).

The ability of the A2B-AR constructs to undergo agonist-induced desensitization was then investigated. Cells were pretreated with 10 μM NECA for 1 h, washed, and subsequently incubated with 10 μM NECA for 20 min, after which cAMP accumulation was determined. In CHO cells expressing WT receptor, NECA pretreatment for 1 h produced an ~50% desensitization in subsequent NECA responsiveness, as compared with cells not preincubated with NECA (Fig. 2B). Similarly, in cells expressing the Leu330-stop receptor mutant, there was marked desensitization of the NECA response. On the other hand, cells expressing the Gln325-stop or Phe328-stop receptor mutants did not display any desensitization subsequent to the 1-h NECA pretreatment. In order to confirm that the differences in desensitization were not due to variations in receptor expression levels, CHO cells were transiently transfected with WT or Gln325-stop A2B-AR constructs. In this case, agonist-stimulated cAMP formation in non-pretreated cells in response to NECA was greater in WT than Gln325-stop cells, but there was still no desensitization in the latter (NECA-stimulated cAMP formation in non-pretreated WT cells was 185 ± 36 and in pretreated cells 62 ± 10 pmol of cyclic AMP mg−1 protein; NECA-stimulated cAMP formation in non-pretreated Gln325-stop cells was 86 ± 23 and in pretreated cells 85 ± 29 pmol of cyclic AMP mg−1 protein). Taken together these results indicate that the Ser329-Leu330 motif close to the COOH terminus of the rat A2B-AR is critical for rapid agonist-induced desensitization. However, the Ser326-stop receptor mutant was also subjected to agonist-induced desensitization, which was unexpected since this mutant does not contain the Ser329-Leu330 motif. We next compared the rates of agonist-stimulated cAMP formation in non-pretreated cells in the presence of a suitable A2B-AR radioligand, an estimation of the cellular distribution of WT receptor and Leu330-stop receptor mutants did not undergo translocation to endosomal compartments. On the other hand, a deep within the cell as had been observed with the WT and Ser326-stop receptor mutants. Instead there appeared to be redistribution of the mutant receptor very close to the cell surface. To confirm the difference in internalization between the WT and Gln325-stop receptor mutant, either WT or Gln325-stop CHO cells were transiently transfected with an A2B-AR-GFP construct which did not contain the NH2 terminus HA-epitope tag (in separate experiments the ability of NECA to stimulate cAMP accumulation in non-transfected CHO cells transiently transfected with either A2B-AR-GFP or HA-tagged
Under basal conditions WT, Gln325-stop, Phe328-stop, and Ser326-stop receptor mutants promoted the rapid translocation of arrestin-2-GFP from cell cytosol to cell membrane upon agonist treatment (Fig. 5; note that coexpression of dyn-DNM did not affect basal cell surface expression of Ser326-stop; arbitrary absorbance units mg⁻¹ cell protein: WT 1.84 ± 0.31 and 1.64 ± 0.25 and Ser326-stop 1.31 ± 0.24 and 1.32 ± 0.23 in the absence or presence of transient dyn-DNM expression, respectively, n = three separate experiments). This indicates that WT and Ser326-stop receptors internalize by different mechanisms; WT receptor internalizes by an arrestin/dynamin/clathrin-dependent mechanism, whereas the Ser326-stop receptor mutant appears to internalize in a dynamin-dependent but arrestin- and clathrin-independent mechanism.

On the basis of the results obtained with the deletion mutants, we engineered and investigated the function of two COOH terminus point mutant receptor constructs, S329G and S326A (Fig. 1). In CHO cells stably expressing these point mutants, NECA stimulated cAMP formation with EC₅₀ values of 0.30 and 1.0 μM for S329G and S326A, respectively (n = three separate experiments), similar to the other constructs studied. Furthermore, relative cell surface receptor expression as assessed by ELISA was similar to WT A₂B-AR expression (arbitrary absorbance units mg⁻¹ cell protein: WT 1.37 ± 0.19; S329G 1.26 ± 0.18; S326A 1.22 ± 0.10; n = three separate experiments for each). As compared with WT A₂B-AR, the S329G mutant was resistant to rapid agonist-induced desensitization (Fig. 8A) and internalization (Fig. 8, B and C). On the other hand, the S326A mutant underwent rapid agonist-induced desensitization and internalization (Fig. 8). Furthermore, the internalized S326A construct co-localized with transferrin (data not shown), indicating that the phenotype of this point mutant is the same as the WT receptor.

**DISCUSSION**

The agonist-induced rapid desensitization and internalization of a number of GPCRs, such as the β₂-adrenoreceptor (25), the AT₁₅-angiotensin receptor (26), and the protease-activated receptor 1 (27) are dependent upon the integrity of the COOH terminus tail of the receptor. In many cases it is likely that GRK-mediated phosphorylation of serine or threonine residues in the receptor leads to non-visual arrestin binding with consequent uncoupling of the receptor from G protein and targeting of the receptor to clathrin-coated pits for internalization (1–5). Although the agonist-induced GRK2- and arrestin-dependent desensitization and internalization of the A₂B-AR has been reported previously (12, 15, 18), it is not known which regions of the receptor are responsible for triggering these processes. Since the intracellular COOH terminus tail plays a critical role in the agonist-induced regulation of a number of GPCRs, we chose in the present study to investigate the role of this region of the receptor in the rapid agonist-induced desensitization and internalization of the rat A₂B-AR.

The deletion mutants (see Fig. 1) were designed primarily to investigate the role of the terminal few amino acids in these processes, since the final 7 amino acids contain 4 potential phosphoacceptor sites (serine/threonine residues), and phos-
Phosphorylation appears to be critical for the desensitization and internalization of many GPCRs (2). In addition, we also examined the Tyr299-stop mutant where the final 33 residues of the COOH terminus had been deleted. However, we were unable to obtain stably transfected CHO cells functionally expressing the latter mutant. When transiently expressed in HEK293 cells, the Tyr299-stop mutant was visualized by confocal microscopy at the cell surface but did not internalize in response to agonist. Other GPCRs with large COOH terminus deletions have also been reported to be non-functional or poorly expressed (28). Apart from the Tyr299-stop mutant, we were able to isolate CHO cell lines stably expressing the WT A2B-AR, as well as the deletion mutants Leu330-stop, Phe328-stop, Ser326-stop, and Gln325-stop. In response to the adenosine receptor agonist NECA, these receptors all coupled to cAMP formation with an EC50 of just under 1 μM, close to the value of 1.4 μM reported for NECA acting at WT human A2B-AR stably expressed in CHO cells (29). However, the maximum response generated for each receptor construct was somewhat different, which may reflect differences in receptor expression between various cell clones. We attempted to assess this with a ligand binding assay using [3H]ZM 241385, a ligand with high affinity for the A2A-AR but which can also label A2B-AR at higher concentrations (Kd 33 nM) (24). However, in preliminary experiments on WT A2B cells we found that very high levels of nonspecific binding made detection of A2B-ARs by this method impossible. Instead, an ELISA assay was employed to assess the relative cell surface expres-

Fig. 5. Agonist-induced cellular redistribution of transiently transfected GFP-tagged WT A2B-AR in CHO cells stably transfected with either WT or Gln325-stop A2B-AR. CHO cells stably expressing WT or Gln325-stop A2B-AR were transiently transfected with 2 μg of eGFP-tagged WT A2B-AR. Two days later cells were preincubated with an anti-HA antibody at 4 °C for 1 h. Subsequently cells were incubated at 37 °C for 30 min in the presence of agonist (NECA; 10 μM). Receptor localization was determined by immunofluorescence in fixed cells as described under “Experimental Procedures.” In these figures HA-tagged and GFP-tagged receptor are red and green, respectively. Marked co-localization (orange/yellow) of stably expressed WT but not Gln325-stop A2B-AR with transiently expressed GFP-A2B-AR is clearly visible in the overlay after agonist stimulation.

Fig. 6. Analysis of agonist-induced translocation of arrestin-2-GFP in CHO cells stably expressing WT or COOH terminus deletion mutant A2B-AR. CHO cells stably expressing WT or COOH terminus deleted mutant A2B-AR were transiently transfected with 0.5 μg of arrestin-2-GFP and then split onto poly-L-lysine-coated coverslips. A2B-AR was visualized using a rhodamine-conjugated anti-HA antibody (all cells shown expressed receptor). Prior to stimulation and viewing, coverslips were mounted in a chamber at 37 °C as described under “Experimental Procedures.” The initial diffuse cytoplasmic distribution of arr-2-GFP is shown prior to agonist stimulation (0 s). NECA (10 μM) was added and the redistribution of arrestins was monitored in real time. Images were obtained at the times shown after agonist addition. Agonist-induced translocation of arrestin-2-GFP was only observed in WT and Leu330-stop mutant receptors.

2 A.-L. Matharu, S. J. Mundell, J. L. Benovic, and E. Kelly, unpublished observations.
Desensitization and Internalization of the A2B-AR

We next examined the ability of the A2B-AR constructs to undergo agonist-induced desensitization and internalization. Since the Leu330-stop mutant desensitizes somewhat more rapidly than the other two constructs, whereas the Ser326-stop mutant is the slowest. Since the internalization of the A2B-AR is arrestin-dependent (15), then it is possible that the inability of the Phe328- and Gln325-stop mutants to undergo rapid agonist-induced desensitization and internalization reflects an inability to interact with arrestins. In previous studies with other GPCRs the ability of mutant receptors to undergo these processes can sometimes be rescued by overexpressing non-visual arrestins in the same cell (30). Consequently, to assess whether the internalization of the Gln325-stop receptor could be rescued by non-visual arrestins, WT or Gln325-stop cells were transiently transfected with GFP-tagged arrestin-2 or arrestin-3. This did not increase the extent of agonist-induced surface receptor loss of WT receptor, indicating that in CHO cells, arrestin levels are not rate-limiting for the maximum extent of internalization. Furthermore, overexpression of GFP-tagged arrestin-2 or arrestin-3 failed to enhance the internalization of the Gln325-stop mutant, suggesting that the distal COOH terminus of the A2B-AR represents a primary site for arrestin interaction.

To further substantiate these results, we directly visualized the receptor constructs by confocal microscopy. Following treatment with agonist for 30 min, and in agreement with the ELISA assays, we found that the WT and Leu330-stop constructs internalized extensively, taking on a punctate distribution consistent with endosomal location, as we have previously reported for the WT receptor (18). On the other hand, the Phe328- and Gln325-stop mutants did not undergo any internalization over the time period examined. The difference between the WT and Gln325-stop mutant was underlined by transiently transfecting WT or Gln325-stop cells with an A2B-AR-GFP construct. Whereas in the WT cells, both WT receptor and A2B-AR-GFP construct underwent internalization whereas the Gln325-stop receptor remained at the cell surface. On the other hand, visualization of the Ser326-stop mutant indicated that this construct underwent limited internalization to a compartment close to the plasma membrane, distinct from that seen with the WT and Leu330-stop constructs.

The ability of GPCR activation to promote the translocation of arrestin-2-GFP from cytosol to cell membrane has been reported for many GPCRs (19) including the A2B-AR (18). To further investigate the interaction of the A2B-AR constructs with non-visual arrestins, we transiently transfected WT and mutant A2B-AR cells with arrestin-2-GFP and visualized translocation of the latter following agonist activation of the receptor. Under basal conditions, arrestin-2-GFP was evenly distributed throughout the cytosol of WT and mutant receptor cells. However, upon agonist activation with 10 μM NECA, arrestin-2-GFP rapidly translocated from cytosol to cell membrane in WT and Leu330-stop cells only. No redistribution of arrestin-2-GFP was observed upon agonist activation of the Phe328-, Ser326-, and Gln325-stop mutants, and thus these constructs appear unable to interact with non-visual arrestins. In order to further identify the region of the A2B-AR COOH terminus which is critical for receptor regulation, we constructed the point mutant S329G. Since this construct was resistant to rapid agonist-induced desensitization/internalization, we can conclude that Ser329, four residues from the extreme COOH terminus of the A2B-AR, is the critical residue for rapid agonist-induced regulation of this receptor. Interestingly, a single threonine residue in the COOH terminus of the canine A2A-AR was found to be critical for rapid agonist-induced desensitization, although internalization was not examined in that study (20).
Although the Ser\textsuperscript{326}-stop receptor mutant undergoes rapid agonist-induced desensitization and internalization as with the WT receptor and Leu\textsuperscript{330}-stop receptor mutant, it apparently does so by an arrestin-independent mechanism. This was confirmed by experiments in which WT and Ser\textsuperscript{326}-stop receptor mutant cells were transiently transfected with dominant negative mutant forms of arrestin-2, dynamin, and Eps15. Arrestin-2-(319–418) contains the clathrin-binding domain of arrestin-2, but not the putative receptor interaction site; it constitutively interacts with clathrin and blocks the internalization of a number of GPCRs including the \( \beta_2\)-AR (31). Dynamin K44A does not express the GTPase activity required in this protein to promote the formation of clathrin-coated vesicles (32), and inhibits the internalization of many GPCRs (19). The Eps15 protein binds to the clathrin adaptor protein AP-2 and is required for normal formation of clathrin-coated pits (33); the EΔ95–295 mutant blocks clathrin-coated pit formation and the internalization of transferrin receptors (34). When these constructs were transiently expressed in WT receptor cells, each markedly reduced the agonist-induced loss of cell surface receptor, confirming the arrestin/dynamin/clathrin dependence of this process, in line with previous findings by ourselves (18) and others (19). Interestingly, although the internalization of the Ser\textsuperscript{326}-stop mutant was blocked by dynamin K44A, it was unaffected by arrestin-2 (319–418) and EΔ95–295, indicating that the agonist-induced internalization of the Ser\textsuperscript{326}-stop mutant is not mediated by arrestins or clathrin-coated pits. Other mechanisms of internalization that are clathrin-independent but dynamin-dependent could include caveolae-mediated internalization (35). To further determine the importance of Ser\textsuperscript{326} in \( \beta_2\)-AR regulation, we constructed a S326A point mutant. However, when stably expressed in CHO cells this mutant displayed the same desensitization/internalization phenotype as the WT and Leu\textsuperscript{330}-stop receptor constructs, indicating that Ser\textsuperscript{326} only directs the receptor into the non-arrestin/clathrin internalization pathway when the TFSLSL motif distal to Ser\textsuperscript{326} is absent. Importantly, these results indicate that care must be taken when interpreting the results of internalization experiments with GPCR COOH terminus mutants; modifications of the COOH terminus can alter not only the rate and extent of internalization, but also the mechanism, as demonstrated here.
In conclusion, we have shown that the rapid agonist-induced desensitization and internalization of the A2B-AR is dependent upon a serine residue (Ser^{329}) close to the end of the receptor COOH terminus. It is likely that this residue represents an arrestin interaction site, and future studies will be directed toward characterizing the possible interaction of non-visual arrestins with this region of the A2B-AR.

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